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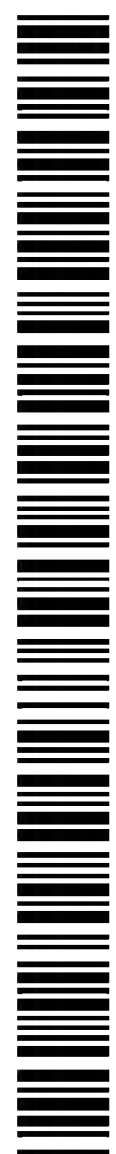
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(54) Title: VACCINE COMPOSITION

(57) Abstract: The present invention relates to the field of vaccine formulation, particularly the field of novel adjuvant compositions comprising outer membrane vesicles (or blebs), and advantageous methods of detoxifying these compositions, and advantageous methods of use of such adjuvants.



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## VACCINE COMPOSITION

### FIELD OF THE INVENTION

The present invention relates to the field of Gram-negative bacterial vaccine compositions, their manufacture, and the use of such compositions in medicine. More particularly it relates to the field of novel adjuvant compositions comprising outer-membrane vesicles (or bleb), and advantageous methods of use of such adjuvants.

### BACKGROUND OF THE INVENTION

Adjuvants are important components in vaccines. Molecules that act as adjuvants may impact on innate immunity, antigen presenting cells (APC) and T lymphocytes. Indeed, by triggering the production of cytokines by macrophages, dendritic cells or natural killer cells, adjuvants will impact on innate immunity. Adjuvants may also stimulate antigen uptake and migration of dendritic cells and macrophages. Finally, adjuvants may also impact on the T-cells cytokine production profile and activate CD4 and/or CD8 T-cells. By impacting on immunity, adjuvants may modify the intrinsic immunogenic properties of an antigen and make this antigen more immunogenic and/or protective.

Although many adjuvant systems are known, there is need to define further, more advantageous adjuvant systems for the production of better vaccines. The present inventors have found bleb preparations in general (and in particular the genetically-modified bleb preparations described herein) are particularly suitable for formulating with other antigens, due to the adjuvant effect they confer on the antigens they are mixed with.

#### *Blebs*

The outer membrane (OM) of Gram-negative bacteria is dynamic and, depending on the environmental conditions, can undergo drastic morphological transformations. Among these manifestations, the formation of outer-membrane vesicles or "blebs" has been studied and documented in many Gram-negative bacteria (Zhou, L *et al.* 1998. *FEMS Microbiol. Lett.* 163: 223-228). Among these, a non-exhaustive list of bacterial pathogens reported to produce blebs include: *Bordetella*

*pertussis*, *Borrelia burgdorferi*, *Brucella melitensis*, *Brucella ovis*, *Chlamydia psittaci*, *Chlamydia trachomatis*, *Escherichia coli*, *Haemophilus influenzae*, *Legionella pneumophila*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa* and *Yersinia enterocolitica*. Although the biochemical mechanism responsible for the production of OM blebs is not fully understood, these outer membrane vesicles have been extensively studied as they represent a powerful methodology in order to isolate outer-membrane protein preparations in their native conformation. In that context, the use of outer-membrane preparations is of particular interest to develop vaccines against *Neisseria*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa* and *Chlamydia*. Moreover, outer membrane blebs combine multiple proteinaceous and non-proteinaceous antigens that are likely to confer extended protection against intra-species variants.

*N. meningitidis* serogroup B (menB) excretes outer membrane blebs in sufficient quantities to allow their manufacture on an industrial scale. Such multicomponent outer-membrane protein vaccines from naturally-occurring menB strains have been found to be efficacious in protecting teenagers from menB disease and have become registered in Latin America. An alternative method of preparing outer-membrane vesicles is via the process of detergent extraction of the bacterial cells (EP 11243).

Examples of bacterial species from which blebs can be made are the following.

*Neisseria meningitidis*:

*Neisseria meningitidis* (meningococcus) is a Gram-negative bacterium frequently isolated from the human upper respiratory tract. It occasionally causes invasive bacterial diseases such as bacteremia and meningitis.

For some years effort have been focused on developing meningococcal outer membrane based vaccines (de Moraes, J.C., Perkins, B., Camargo, M.C. et al. Lancet 340: 1074-1078, 1992; Bjune, G., Hoiby, E.A. Gronnesby, J.K. et al. 338: 1093-1096, 1991). Such vaccines have demonstrated efficacies from 57% - 85% in older children (>4 years) and adolescents. Most of these efficacy trials were performed with OMV (outer membrane vesicles, derived by LPS depletion from blebs) vaccines derived from wild-type *N. meningitidis* B strains.

Many bacterial outer membrane components are present in these vaccines, such as PorA, PorB, Rmp, Opc, Opa, FrpB and the contribution of these components to the observed protection still needs further definition. Other bacterial outer membrane components have been defined (using animal or human antibodies) as potentially being relevant to the induction of protective immunity, such as TbpB, NspA (Martin, D., Cadieux, N., Hamel, J., Brodeux, B.R., J. Exp. Med. 185: 1173-1183, 1997; Lissolo, L., Maître-Wilmotte, C., Dumas, p. et al., Inf. Immun. 63: 884-890, 1995). The mechanism of protective immunity will involve antibody mediated bactericidal activity and opsonophagocytosis.

#### *Moraxella catarrhalis*

*Moraxella catarrhalis* (also named *Branhamella catarrhalis*) is a Gram-negative bacterium frequently isolated from the human upper respiratory tract. It is responsible for several pathologies, the main ones being otitis media in infants and children, and pneumonia the elderly. It is also responsible for sinusitis, nosocomial infections and, less frequently, for invasive diseases.

*M. catarrhalis* produces outer membrane vesicles (Blebs). These Blebs have been isolated or extracted by using different methods (Murphy T.F., Loeb M.R. 1989. Microb. Pathog. 6: 159-174; Unhanand M., Maciver, I., Ramillo O., Arencibia-Mireles O., Argyle J.C., McCracken G.H. Jr., Hansen E.J. 1992. J. Infect. Dis. 165:644-650). The protective capacity of such Bleb preparations has been tested in a murine model for pulmonary clearance of *M. catarrhalis*. It has been shown that active immunization with Bleb vaccine or passive transfer of anti-Blebs antibody induces significant protection in this model (Maciver I., Unhanand M., McCracken G.H. Jr., Hansen, E.J. 1993. J. Infect. Dis. 168: 469-472). Proteins present on the surface of *M. catarrhalis* have been characterized using biochemical methods for their potential implication in the induction of a protective immunity (for review, see Murphy, TF (1996) Microbiol.Rev. 60:267) e.g. OMP B1, a 84 kDa protein, the expression of which is regulated by iron, and that is recognized by the sera of patients with pneumonia (Sethi, S, et al. (1995) Infect.Immun. 63:1516), and of UspA1 and UspA2 (Chen D. et al.(1999), Infect.Immun. 67:1310). In a mouse pneumonia model, the presence of antibodies raised against some of them (UspA, CopB) favors a faster clearance of the pulmonary infection. Another polypeptide (OMP CD) is highly



conserved among *M. catarrhalis* strains, and presents homologies with a porin of *Pseudomonas aeruginosa*, which has been demonstrated to be efficacious against this bacterium in animal models.

## 5 *Haemophilus influenzae*

*Haemophilus influenzae* is a non-motile Gram-negative bacterium. Man is its only natural host. *H. influenzae* isolates are usually classified according to their polysaccharide capsule. Six different capsular types designated 'a' through 'f' have been identified. Isolates that fail to agglutinate with antisera raised against one of these six  
10 serotypes are classified as nontypeable, and do not express a capsule.

*H. influenzae* type b (Hib) is clearly different from the other types in that it is a major cause of bacterial meningitis and systemic diseases. Nontypeable strains of *H. influenzae* (NTHi) are only occasionally isolated from the blood of patients with systemic disease. NTHi is a common cause of pneumonia, exacerbation of chronic  
15 bronchitis, sinusitis and otitis media. NTHi strains demonstrate a large variability as identified by clonal analysis, whilst Hib strains as a whole are more homogeneous.

Outer membrane vesicles (or blebs) have been isolated from *H. influenzae* (Loeb M.R., Zachary A.L., Smith D.H. 1981. J. Bacteriol. 145:569-604; Stull T.L., Mack K., Haas J.E., Smit J., Smith A.L. 1985. Anal. Biochem. 150: 471-480). The  
20 vesicles have been associated with the induction of blood-brain barrier permeability (Wiwpelwey B., Hansen E.J., Scheld W.M. 1989 Infect. Immun. 57: 2559-2560), the induction of meningeal inflammation (Mustafa M.M., Ramilo O., Syrogiannopoulos G.A., Olsen K.D., McCracken G.H. Jr., Hansen, E.J. 1989. J. Infect. Dis. 159: 917-922) and to DNA uptake (Concino M.F., Goodgal S.H. 1982 J. Bacteriol. 152: 441-450).  
25 These vesicles are able to bind and be absorbed by the nasal mucosal epithelium (Harada T., Shimuzu T., Nishimoto K., Sakakura Y. 1989. Acta Otorhinolarygol. 246: 218-221) showing that adhesins and/or colonization factors could be present in Blebs. Immune response to proteins present in outer membrane vesicles has been observed in patients with various *H. influenzae* diseases (Sakakura Y., Harada T., Hamaguchi Y.,  
30 Jin C.S. 1988. Acta Otorhinolarygol. Suppl. (Stockh.) 454: 222-226; Harada T., Sakakura Y., Miyoshi Y. 1986. Rhinology 24: 61-66).

Various surface-exposed proteins of *H. influenzae* have been shown to be involved in pathogenesis or have been shown to confer protection upon vaccination in animal models.

For instance various adhesins have been found (fimbriae and pili [Brinton CC. et al. 1989. *Pediatr. Infect. Dis. J.* 8:S54; Kar S. et al. 1990. *Infect. Immun.* 58:903; Gildorf JR. et al. 1992. *Infect. Immun.* 60:374; St. Geme JW et al. 1991. *Infect. Immun.* 59:3366; St. Geme JW et al. 1993. *Infect. Immun.* 61: 2233], HMW1 and HMW2 [St. Geme JW. et al. 1993. *Proc. Natl. Acad. Sci. USA* 90:2875], NTHi 115-kDa Hia protein [Barenkamp SJ., St Geme S.W. 1996. *Mol. Microbiol.*] which is highly similar to *H. influenzae* type b Hsf [St. Geme JW. et al. 1996. *J. Bact.* 178:6281], and Hap [St. Geme JW. et al. 1994. *Mol. Microbiol.* 14:217].

Five major outer membrane proteins (OMP) have also been identified: P1, 2, 3, 4 and 5 (Loeb MR. et al. 1987. *Infect. Immun.* 55:2612; Musson RS. Jr. et al. 1983. *J. Clin. Invest.* 72:677; Haase EM. et al. 1994 *Infect. Immun.* 62:3712; Troelstra A. et al. 1994 *Infect. Immun.* 62:779; Green BA. et al. 1991. *Infect. Immun.* 59:3191). OMP P6 is a conserved peptidoglycan associated lipoprotein making up 1-5 % of the outer membrane (Nelson MB. et al. 1991. *Infect. Immun.* 59:2658; Demaria TF. et al. 1996. *Infect. Immun.* 64:5187).

In line with the observations made with gonococci and meningococci, NTHi expresses on its surface a dual human transferrin receptor composed of TbpA and TbpB when grown under iron limitation (Loosmore SM. et al. 1996. *Mol. Microbiol.* 19:575). Hemoglobin / haptoglobin receptor also have been described for NTHi (Maciver I. et al. 1996. *Infect. Immun.* 64:3703). A receptor for Haem:Hemopexin has also been identified (Cope LD. et al. 1994. *Mol. Microbiol.* 13:868). A lactoferrin receptor is also present amongst NTHi (Schryvers AB. et al. 1989. *J. Med. Microbiol.* 29:121).

Other interesting antigens on the surface of the bacterium include an 80kDa OMP, the D15 surface antigen (Flack FS. et al. 1995. *Gene* 156:97); a 42kDa outer membrane lipoprotein, LPD (Akkoyunlu M. et al. 1996. *Infect. Immun.* 64:4586); a minor 98kDa high molecular weight adhesin OMP (Kimura A. et al. 1985. *Infect. Immun.* 47:253); IgA1-protease (Mulks MH., Shoberg RJ. 1994. *Meth. Enzymol.*

235:543); OMP26 (Kyd, J.M., Cripps, A.W. 1998. Infect. Immun. 66:2272); and NTHi HtrA protein (Loosmore S.M. et al. 1998. Infect. Immun. 66:899).

*Pseudomonas aeruginosa*:

5           The genus *Pseudomonas* consists of Gram-negative, polarly flagellated, straight and slightly curved rods that grow aerobically and do not form spores. Because of their limited metabolic requirements, *Pseudomonas spp.* are ubiquitous and are widely distributed in the soil, the air, sewage water and in plants. Numerous species of *Pseudomonas* such as *P. aeruginosa*, *P. pseudomallei*, *P. mallei*, *P.*  
10 *maltophilia* and *P. cepacia* have also been shown to be pathogenic for humans. Among this list, *P. aeruginosa* is considered as an important human pathogen since it is associated with opportunistic infection of immuno-compromised host and is responsible for high morbidity in hospitalized patients. Nosocomial infection with *P. aeruginosa* afflicts primarily patients submitted for prolonged treatment and receiving  
15 immuno-suppressive agents, corticosteroids, antimetabolites antibiotics or radiation.

          To examine the protective properties of OM proteins, a vaccine containing *P. aeruginosa* OM proteins of molecular masses ranging from 20 to 100 kDa has been used in pre-clinical and clinical trials. This vaccine was efficacious in animal models against *P. aeruginosa* challenge and induced high levels of specific antibodies in  
20 human volunteers. Plasma from human volunteers containing anti-*P. aeruginosa* antibodies provided passive protection and helped the recovery of 87% of patients with severe forms of *P. aeruginosa* infection. More recently, a hybrid protein containing parts of the outer membrane proteins OprF (amino acids 190-342) and OprI (amino acids 21-83) from *Pseudomonas aeruginosa* fused to the glutathione-S-  
25 transferase was shown to protect mice against a 975-fold 50% lethal dose of *P. aeruginosa* (Knapp et al. 1999. Vaccine. 17:1663-1669).

          The present inventors have realised that blebs may be used as an effective adjuvant in conjunction with antigens.

30           Although wild-type blebs may be used, the inventors have realised a number of drawbacks associated with the use of wild-type blebs (either naturally occurring or chemically made).

Examples of such problems are the following:

- the toxicity of the LPS remaining on the surface of the bleb
- the potential induction of an autoimmune response because of host-identical structures (for example the capsular polysaccharide in *Neisseria meningitidis* serogroup B, the lacto-N-neotetraose in *Neisseria* LPS, saccharide structure within ntHi LPS, saccharide structures within Pili).
- the presence of immunodominant but variable proteins on the bleb (PorA; TbpB, Opa [*N. meningitidis* B]; P2, P5 [non-typeable *H. influenzae*]) – such blebs being effective only against a restricted selection of bacterial species. Type-specificity of the bactericidal antibody response may preclude the use of such vaccines in infancy.
- the presence of unprotective (non relevant) antigens (Rmp, H8, ...) on the bleb – antigens that are decoys for the immune system
- the lack of presence of important molecules which are produced conditionally (for instance iron-regulated outer membrane proteins, IROMP's, in vivo regulated expression mechanisms) – such conditions are hard to control in bleb production in order to optimise the amount of antigen on the surface
- the low level of expression of protective, (particularly conserved) antigens (NspA, P6)

Although the first 2 problems are troublesome to use certain bleb preparations as adjuvants, the latter 4 problems are troublesome if the bleb is also to be included in a vaccine in its own right as an immunogenic component against the bacteria from which it is derived.

Such problems may prevent the use of bleb components in human vaccine reagents. This is particularly so for paediatric use (<4 years) where reactogenicity against bleb components is particularly important, and where bleb vaccines (for instance the previously mentioned marketed MenB bleb vaccine) have been shown to be ineffective at immuno-protecting.

Accordingly, the present invention provides methods of alleviating the above problems using genetically engineered bacterial strains, which result in improved bleb



adjuvants. Such methods will be especially useful in the generation of new vaccines against bacterial pathogens such as *Neisseria meningitidis*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and others.

Each of these methods of improvement individually improve the bleb adjuvant, however a combination of one or more of these methods work in conjunction so as to produce an optimised engineered bleb vaccine component which is non-toxic, with a strong adjuvant activity, suitable for paediatric use, and which may be immuno-protective in its own right against the organism from which it is derived.

## SUMMARY OF THE INVENTION

The present invention provides various uses of Gram-negative bacterial blebs as an effective adjuvant in immunogenic compositions.

In one embodiment there is provided an immunogenic composition comprising an antigen derived from a pathogen which is capable of protecting a host against said pathogen, mixed with an adjuvant comprising a bleb preparation derived from a Gram-negative bacterial strain.

Preferably the bacterial source of the bleb adjuvant is from a difference strain or species than the source of the antigen (they are heterologous). Most preferably they are from different pathogens. Preferred compositions are made by adding blebs and antigen to the formulation separately.

The antigen may be a polysaccharide or polysaccharide conjugate antigen. In such case a composition consisting of *N. meningitidis* B bleb and *N. meningitidis* C polysaccharide (as described in WO 99/61053) is not included in the invention.

Alternatively, the antigen may be a peptide or protein antigen.

The inventors have realised that bleb adjuvants are particularly useful where a fast-acting protective immune response against the antigen is required. Blebs can be particularly useful in this regard over other adjuvants. A method of inducing a fast-acting protective immune response against the antigen contained in the immunogenic compositions of the invention is also provided, comprising the step of administering to a host an effective amount of the immunogenic composition of the invention. This is particularly useful in vaccines for the elderly, thus a method of protecting an elderly

patient against a pathogen by administering to said patient an effective amount of the immunogenic composition of the invention in which the antigen is derived from said pathogen, and the use of the adjuvant in this regard are further provided.

The blebs of the invention may be a wild-type preparation (collected from the bacterial culture or extracted with detergent such as deoxycholate), or may be a genetically-engineered bleb preparation from a Gram-negative bacterial strain characterized in that said preparation is obtainable by employing one or more processes selected from the following group:

- a) a process of reducing immunodominant variable or non-protective antigens within the bleb preparation comprising the steps of determining the identity of such antigen, engineering a bacterial strain to produce less or none of said antigen, and making blebs from said strain;
- b) a process of upregulating expression of protective, endogenous (and preferably conserved) OMP antigens within the bleb preparation comprising the steps of identifying such antigen, engineering a bacterial strain so as to introduce a stronger promoter sequence upstream of a gene encoding said antigen such that said gene is expressed at a level higher than in the non-modified bleb, and making blebs from said strain;
- c) a process of upregulating expression of conditionally-expressed, protective (and preferably conserved) OMP antigens within the bleb preparation comprising the steps of identifying such an antigen, engineering a bacterial strain so as to remove the repressive control mechanisms of its expression (such as iron restriction), and making blebs from said strain;
- d) a process of modifying lipid A portion of bacterial LPS within the bleb preparation, comprising the steps of identifying a gene involved in rendering the lipid A portion of LPS toxic, engineering a bacterial strain so as to reduce or switch off expression of said gene, and making blebs from said strain;
- e) a process of modifying lipid A portion of bacterial LPS within the bleb preparation, comprising the steps of identifying a gene involved in rendering the lipid A portion of LPS less toxic, engineering a bacterial strain so as to introduce a stronger promoter sequence upstream of said

gene such that said gene is expressed at a level higher than in the non-modified bleb, and making blebs from said strain;

f) a process of reducing lipid A toxicity within the bleb preparation and increasing the levels of protective antigens, comprising the steps of engineering the chromosome of a bacterial strain to incorporate a gene encoding a Polymyxin A peptide, or a derivative or analogue thereof, fused to a protective antigen, and making blebs from said strain;

g) a process of creating conserved OMP antigens on the bleb preparation comprising the steps of identifying such antigen, engineering a bacterial strain so as to delete variable regions of a gene encoding said antigen, and making blebs from said strain;

h) a process of reducing expression within the bleb preparation of an antigen which shares a structural similarity with a human structure and may be capable of inducing an auto-immune response in humans (such as the capsular polysaccharide of *N. meningitidis* B), comprising the steps of identifying a gene involved in the biosynthesis of the antigen, engineering a bacterial strain so as to reduce or switch off expression of said gene, and making blebs from said strain; or

i) a process of upregulating expression of protective, endogenous (and preferably conserved) OMP antigens within the bleb preparation comprising the steps of identifying such antigen, engineering a bacterial strain so as to introduce into the chromosome one or more further copies of a gene encoding said antigen controlled by a heterologous, stronger promoter sequence, and making blebs from said strain.

Processes d), e), f) and h) are particularly advantageous in the manufacture of bleb adjuvants of the invention that are safe in humans. One or more (2, 3, or 4) of these processes are preferably used to manufacture bleb adjuvant.

In a specific embodiment the immunogenic composition of the invention may thus comprise a bleb adjuvant made by process d) wherein the bleb preparation is derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to reduce or switch off expression of one or more

genes selected from the group consisting of: htrB, msbB and lpxK (or homologues thereof).

In a further embodiment the immunogenic composition of the invention may comprise a bleb adjuvant made by process e) wherein the bleb preparation is derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to express at a higher level one or more genes selected from the group consisting of: pmrA, pmrB, pmrE and pmrF (or homologues thereof).

In a still further embodiment the immunogenic composition of the invention may comprise a bleb adjuvant made by process h) wherein the bleb preparation is derived from a strain engineered not produce a capsular polysaccharide, lipopolysaccharide or lipooligosaccharide comprising an antigen similar to a human structure by reducing or switching off expression of one or more genes selected from the group consisting of: galE, siaA, siaB, siaC, siaD, ctrA, ctrB, ctrC and ctrD (or homologues thereof).

## BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1:** Reactivity of the 735 mAb on different colonies.

**Figure 2:** Reactivities of specific monoclonal antibodies by whole cell Elisa.

**Figure 3:** Schematic representation of the pCMK vectors used to deliver genes, operons and/or expression cassettes in the genome of *Neisseria meningitidis*.

**Figure 4:** Analysis of PorA expression in total protein extracts of recombinant *N. meningitidis* serogroupB (H44/76 derivatives). Total proteins were recovered from cps- (lanes 3 and 4), cps- porA::pCMK+ (lanes 2 and 5) and cps- porA::nspA (lanes 1 and 6) recombinant *N. meningitidis* serogroupB strains and were analyzed under SDS-PAGE conditions in a 12% polyacrylamide gel. Gels were stained with Coomassie blue (lanes 1 to 3) or transferred to a nitrocellulose membrane and immuno-stained with an anti-PorA monoclonal antibody.



**Figure 5:** Analysis of NspA expression in protein extracts of recombinant *N. meningitidis* serogroupB strains (H44/76 derivatives). Proteins were extracted from whole bacteria (lanes 1 to 3) or outer-membrane blebs preparations (lanes 4 to 6) separated by SDS-PAGE on a 12% acrylamide gel and analyzed by immuno-blotting using an anti-NspA polyclonal serum. Samples corresponding to *cps*- (lanes 1 and 6), *cps*- *porA*::*pCMK*+ (lanes 3 and 4) and *cps*- *porA*::*nspA* (lanes 2 and 5) were analyzed. Two forms of NspA were detected: a mature form (18kDa) co-migrating with the recombinant purified NspA, and a shorter form (15kDa).

**Figure 6:** Analysis of D15/omp85 expression in protein extracts of recombinant *N. meningitidis* serogroupB strains (H44/76 derivatives). Proteins were extracted from outer-membrane blebs preparations and were separated by SDS-PAGE on a 12% acrylamide gel and analyzed by immuno-blotting using an anti-omp85 polyclonal serum. Samples corresponding to *cps*- (lane 2), and *cps*-, *PorA*+, *pCMK*+*Omp85/D15* (lane 1) recombinant *N. meningitidis* serogroupB strains were analyzed.

**Figure 7:** General strategy for modulating gene expression by promoter delivery (RS stands for restriction site).

**Figure 8:** Analysis of outer-membrane blebs produced by recombinant *N. meningitidis* serogroupB *cps*- strains (H44/76 derivatives). Proteins were extracted from outer-membrane bleb preparations and were separated by SDS-PAGE under reducing conditions on a 4-20% gradient polyacrylamide gel. The gel was stained with Coomassie brilliant blue R250. Lanes 2, 4, 6 corresponded to 5µg of total proteins whereas lanes 3, 5 and 7 were loaded with 10µg proteins.

**Figure 9:** Construction of a promoter replacement plasmid used to up-regulate the expression/production of Omp85/D15 in *Neisseria meningitidis* H44/76.

**Figure 10:** Analysis of OMP85 expression in total protein extracts of recombinant *NmB* (H44/76 derivatives). Gels were stained with Coomassie blue (A) or transferred

to nitrocellulose membrane and immuno-stained with rabbit anti-OMP85 (*N.gono*) monoclonal antibody (B).

**Figure 11:** Analysis of OMP85 expression in OMV preparations from recombinant *NmB* (H44/76 derivatives). Gels were stained with Coomassie blue (A) or transferred to nitrocellulose membrane and immuno-stained with rabbit anti-OMP85 polyclonal antibody (B).

**Figure 12:** Schematic representation of the recombinant PCR strategy used to delete the *lacO* in the chimeric *porA/lacO* promoter.

**Figure 13:** Analysis of Hsf expression in total protein extracts of recombinant *N. meningitidis* serogroup B (H44/76 derivatives). Total proteins were recovered from Cps-PorA+(lanes 1), and Cps-PorA+/Hsf (lanes 2) recombinant *N. meningitidis* serogroup B strains and were analyzed under SDS-PAGE conditions in a 12% polyacrylamide gel. Gels were stained with Coomassie blue .

**Figure 14:** Analysis of GFP expression in total protein extracts of recombinant *N. meningitidis* (H44/76 derivative). Total protein were recovered from Cps-, PorA+ (lane1), Cps-, PorA- GFP+ (lane2 & 3) recombinant strains. Proteins were separated by PAGE-SDS in a 12% polyacrylamide gel and then stained with Coomassie blue.

**Figure 15:** Illustration of the pattern of major proteins on the surface of various recombinant bleb preparations as analysed by SDS-PAGE (Coomassie staining).

**Figure 16:** Specific anti-Hsf response for various bleb and recombinant bleb preparations using purified recombinant Hsf protein.

**Figure 17:** Analysis of NspA expression in total protein extracts of recombinant *NmB* (serogroup B derivatives). Gels were stained with Coomassie blue (A) or transferred to nitrocellulose membrane and immuno-stained with mouse anti-PorA monoclonal antibody (B) or mouse anti-NspA polyclonal antibody (C).

## DESCRIPTION OF THE INVENTION

### 5 Vaccine Combinations & Advantageous Uses of blebs as Adjuvants

Immunogenic compositions of the invention (preferably vaccine combinations) may comprise wild-type Gram-negative bacterial bleb preparations (isolated from the culture medium, or from cells by detergent [e.g. deoxycholate] extraction) or the genetically-modified bleb preparations described later. The antigen  
10 against a disease state is preferably from a heterologous source from the source of the blebs, and is preferably mixed with the bleb in the composition rather than having been expressed on its surface.

It has also been found that when antigens are formulated with a bleb adjuvant in a vaccine in this way, this vaccine may induce a faster immune response against the  
15 antigen (as well as a larger response). The adjuvant may therefore be particularly suitable for vaccines for the elderly (over 55 years of age).

The present invention provides an immunogenic composition comprising an antigen derived from a pathogen which is capable of protecting a host against said pathogen, mixed with an adjuvant comprising a bleb preparation derived from a  
20 Gram-negative bacterial strain. Although the source of the antigen and the bleb are preferably heterologous, they may still be derived from the same class of pathogen: for instance the antigen may be 1 or more (2 or 3) meningococcal capsular polysaccharides (plain or preferably conjugated) selected from a group comprising: A, Y or W (optionally also comprising group C conjugate), and the bleb preparation  
25 may be from a meningococcus B strain. Such a vaccine may be advantageously used as a global meningococcus vaccine.

By conjugated it is meant that the antigen is covalently linked to a protein which is a source of T-helper epitopes such as tetanus toxoid, diphtheria toxoid, CRM197, pneumococcal pneumolysin, protein D from *H. influenzae*, or OmpC from  
30 meningococcus. When an antigen is conjugated the immunogenicity and the protective capacity of either or both the antigen and the carrier (against the organisms from which they are derived) may be significantly enhanced.

In a further embodiment, the antigen and the Gram-negative bacterial bleb preparation may be from different pathogens. For instance, the antigen may be a *H. influenzae* antigen (either a protein [as described below] or preferably a conjugated capsular polysaccharide from *H. influenzae* b), and the bleb preparation from meningococcus B. If both a conjugated capsular polysaccharide from *H. influenzae* b and two or more conjugated meningococcal capsular polysaccharides (selected from A, C, Y and W) are included, such a vaccine may advantageously constitute a global meningitis vaccine (particularly if pneumococcal antigens are also included as described below).

Alternatively, the antigen is one or more capsular polysaccharide(s) from *Streptococcus pneumoniae* (plain or preferably conjugated), and/or one or more protein antigens that is capable of protecting a host against *Streptococcus pneumoniae* infection, and the bleb preparation is from meningococcus B.

The pneumococcal capsular polysaccharide antigens are preferably selected from serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F (most preferably from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F).

Preferred pneumococcal proteins antigens are those pneumococcal proteins which are exposed on the outer surface of the pneumococcus (capable of being recognised by a host's immune system during at least part of the life cycle of the pneumococcus), or are proteins which are secreted or released by the pneumococcus. Most preferably, the protein is a toxin, adhesin, 2-component signal transducer, or lipoprotein of *Streptococcus pneumoniae*, or fragments thereof. Particularly preferred proteins include, but are not limited to: pneumolysin (preferably detoxified by chemical treatment or mutation) [Mitchell *et al.* Nucleic Acids Res. 1990 Jul 11; 18(13): 4010 "Comparison of pneumolysin genes and proteins from *Streptococcus pneumoniae* types 1 and 2.", Mitchell *et al.* Biochim Biophys Acta 1989 Jan 23; 1007(1): 67-72 "Expression of the pneumolysin gene in *Escherichia coli*: rapid purification and biological properties.", WO 96/05859 (A. Cyanamid), WO 90/06951 (Paton *et al.*), WO 99/03884 (NAVA)]; PspA and transmembrane deletion variants thereof (US 5804193 - Briles *et al.*); PspC and transmembrane deletion variants thereof (WO 97/09994 - Briles *et al.*); PsaA and transmembrane deletion variants



thereof (Berry & Paton, Infect Immun 1996 Dec;64(12):5255-62 "Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*"; pneumococcal choline binding proteins and transmembrane deletion variants thereof; CbpA and transmembrane deletion variants thereof (WO 97/41151; WO 99/51266); Glyceraldehyde-3-phosphate – dehydrogenase (Infect. Immun. 1996 64:3544); HSP70 (WO 96/40928); PcpA (Sanchez-Beato et al. *FEMS Microbiol Lett* 1998, 164:207-14); M like protein, SB patent application No. EP 0837130; and adhesin 18627, SB Patent application No. EP 0834568. Further preferred pneumococcal protein antigens are those disclosed in WO 98/18931, particularly those selected in WO 98/18930 and PCT/US99/30390 (incorporated by reference herein).

The above mentioned meningococcal blebs may be from a wild-type strain, or might be a mixture from 2 or more (preferably several) wild-type strains belonging to several subtype/serotypes (for instance chosen from P1.15, P1.7,16, P1.4, and P1.2).

The above mentioned meningococcal blebs may also be genetically engineered to improve them in a way discussed below. Preferably, the meningococcus B bleb preparation is derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to reduce or switch off expression of one or more genes selected from the group consisting of: htrB, msbB and lpxK (or homologues thereof).

By 'reduce' it is meant that expression from a gene has been decreased by 10, 20, 30, 40, 50, 60, 70, 80, or 90%. By 'switch off' it is meant the gene is deleted from the genome or in some other way produces no active gene product.

Alternatively, or in combination, the meningococcal B bleb preparation is derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to express at a higher level one or more genes selected from the group consisting of: pmrA, pmrB, pmrE and pmrF.

By 'express at a higher level' it is meant that more than 10, 30, 50, 70, 90, 150, 300% additional gene product is made by the recombinant bacterium than in the wild-type strain.

A further improvement which may be an alternative or in combination with either or both of the previous improvements is that the meningococcal B bleb

preparation is derived from a strain which does not produce B capsular polysaccharide, due to the strain having been engineered to reduce or switch off expression of one or more genes selected from the group consisting of: galE, siaA, siaB, siaC, siaD, ctrA, ctrB, ctrC and ctrD (or homologues thereof). These mutations  
5 may also remove human-like epitopes from the LOS of the bleb.

*Compositions useful for the treatment of otitis media*

In a further embodiment the antigen in the immunogenic composition is from *H. influenzae*, and the bleb preparation is from *Moraxella catarrhalis*. The antigen  
10 may be a conjugated capsular polysaccharide from *H. influenzae* b, or may be one or more protein antigens that can protect a host against non-typeable *H. influenzae* infection.

Preferred non-typeable *H. influenzae* protein antigens include Fimbrin protein (US 5766608) and fusions comprising peptides therefrom (eg LB1 Fusion) (US  
15 5843464 - Ohio State Research Foundation), OMP26, P6, protein D, TbpA, TbpB, Hia, Hmw1, Hmw2, Hap, and D15.

Alternatively, the antigen may be from *Streptococcus pneumoniae*, and the bleb preparation from *Moraxella catarrhalis*. The pneumococcal antigen may be one or more capsular polysaccharide(s) (preferably conjugated) from *Streptococcus*  
20 *pneumoniae* (as described above), and/or one or more proteins from *Streptococcus pneumoniae* capable of protecting a host against pneumococcal disease (as described above).

The above immunogenic compositions comprising a *Moraxella catarrhalis* bleb preparation adjuvant may also optionally comprise one or more antigens that can  
25 protect a host against RSV and/or one or more antigens that can protect a host against influenza virus.

Preferred influenza virus antigens include whole, live or inactivated virus, split influenza virus, grown in eggs or MDCK cells, or Vero cells or whole flu virosomes (as described by R. Gluck, Vaccine, 1992, 10, 915-920) or purified or recombinant  
30 proteins thereof, such as HA, NP, NA, or M proteins, or combinations thereof.

Preferred RSV (Respiratory Syncytial Virus) antigens include the F glycoprotein, the G glycoprotein, the HN protein, or derivatives thereof.

In a preferred embodiment, the *Moraxella catarrhalis* bleb adjuvant is formulated with one or more plain or conjugated pneumococcal capsular polysaccharides, and one or more antigens that can protect a host against non-typeable *H. influenzae* infection (as defined above). Optionally, the vaccine may also comprise one or more protein antigens that can protect a host against *Streptococcus pneumoniae* infection (as defined above). The vaccine may also optionally comprise one or more antigens that can protect a host against RSV and/or one or more antigens that can protect a host against influenza virus (as defined above). Such a vaccine may be advantageously used as a global otitis media vaccine.

The *Moraxella catarrhalis* bleb adjuvant mentioned above may be derived from a wild-type strain, or might be a mixture from 2 or more (preferably several) wild-type strains belonging to several subtype/serotypes.

The above mentioned *Moraxella catarrhalis* bleb adjuvant may also be genetically engineered to improve the blebs in a way discussed below. Preferably, the *Moraxella catarrhalis* bleb preparation is derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to reduce or switch off expression of one or more genes selected from the group consisting of: htrB, msbB and lpxK (or homologues thereof).

Alternatively, or in combination with the above improvement, the *Moraxella catarrhalis* bleb adjuvant is derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to express at a higher level one or more genes selected from the group consisting of: pmrA, pmrB, pmrE and pmrF.

A further improvement which may be an alternative or in combination with either or both of the previous improvements is that the *Moraxella catarrhalis* bleb adjuvant is derived from a strain which has been engineered to remove human-like epitopes from the LPS of the bleb. This could be done, for instance, by the strain having been engineered to reduce or switch off expression of one or more genes selected from the group consisting of: galE, siaA, siaB, siaC, siaD, ctrA, ctrB, ctrC and ctrD (or homologues thereof).

In a still further embodiment the antigen in the immunogenic composition is a conjugated capsular polysaccharide from *H. influenzae* b, and the bleb preparation is from non-typeable *H. influenzae*.

Alternatively, the antigen may be from *Streptococcus pneumoniae*, and the  
5 bleb preparation from non-typeable *H. influenzae*. The pneumococcal antigen may be one or more capsular polysaccharide(s) (preferably conjugated) from *Streptococcus pneumoniae* (as described above), and/or one or more proteins from *Streptococcus pneumoniae* capable of protecting a host against pneumococcal disease (as described above).

10 Alternatively, the antigen may be from *Moraxella catarrhalis* (preferably one or more proteins from *M. catarrhalis* capable of protecting a host against disease caused by this organism [most preferably one of the protective antigens mentioned above or mentioned below as being usefully upregulated in a *Moraxella catarrhalis* bleb vaccine]) and the bleb preparation from non-typeable *H. influenzae*.

15 The above immunogenic compositions comprising a non-typeable *H. influenzae* bleb preparation adjuvant may also optionally comprise one or more antigens that can protect a host against RSV (as described above) and/or one or more antigens that can protect a host against influenza virus (as described above).

In a preferred embodiment, the non-typeable *H. influenzae* bleb adjuvant is  
20 formulated with one or more plain or conjugated pneumococcal capsular polysaccharides, and one or more antigens that can protect a host against *M. catarrhalis* infection (as defined above). Optionally, the vaccine may also comprise one or more protein antigens that can protect a host against *Streptococcus pneumoniae* infection (as defined above). The vaccine may also optionally comprise one or more  
25 antigens that can protect a host against RSV and/or one or more antigens that can protect a host against influenza virus (as defined above). Such a vaccine may be advantageously used as a global otitis media vaccine.

The non-typeable *H. influenzae* bleb adjuvant mentioned above may be derived from a wild-type strain, or might be a mixture from 2 or more (preferably  
30 several) wild-type strains belonging to several subtype/serotypes.

The above mentioned non-typeable *H. influenzae* bleb adjuvant may also be genetically engineered to improve the blebs in a way discussed below. Preferably, the



non-typeable *H. influenzae* bleb preparation is derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to reduce or switch off expression of one or more genes selected from the group consisting of: htrB, msbB and lpxK.

5           Alternatively, or in combination with the above improvement, the non-typeable *H. influenzae* bleb adjuvant is derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to express at a higher level one or more genes selected from the group consisting of: pmrA, pmrB, pmrE and pmrF.

10           A further improvement which may be an alternative or in combination with either or both of the previous improvements is that the *H. influenzae* bleb adjuvant is derived from a strain which has been engineered to remove human-like epitopes from the LPS of the bleb. This could be done, for instance, by the strain having been engineered to reduce or switch off expression of one or more genes selected from the  
15           group consisting of: galE, siaA, siaB, siaC, siaD, ctrA, ctrB, ctrC and ctrD (or homologues thereof).

A further aspect of the invention is a vaccine composition comprising the above immunogenic compositions of the invention, and a pharmaceutically acceptable  
20           excipient or carrier. Preferable such vaccines should be formulated as described below in "vaccine formulations".

The amount of polysaccharide antigen (plain or in a conjugate) in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccines. Such amount will vary depending  
25           upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 0.1-100 µg of polysaccharide, preferably 0.1-50 µg, preferably 0.1-10 µg, of which 1 to 5 µg is the most preferable range.

The content of protein antigens in the vaccine will typically be in the range 1-100µg, preferably 5-50µg, most typically in the range 5 - 25µg. The amount of bleb  
30           adjuvant present in the formulations should be present in a similar range of quantity.

Optimal amounts of components for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects.

Following an initial vaccination, subjects may receive one or several booster immunisations adequately spaced.

The immunogenic compositions or vaccines of this aspect of the invention  
5 may have one or more of the following advantages: i) higher immune response against the antigen; ii) higher protective capacity of the antigen; iii) faster immune response against the antigen; iv) faster protection by the antigen; v) where the antigen is a conjugated polysaccharide antigen, i) ii), iii) or iv) may apply to both the polysaccharide and the carrier; vi) the antigen may enhance the immune response or  
10 protective capacity of a protective antigen present on the surface of the bleb preparation.

A further embodiment of this aspect of the invention is a method of inducing a faster immune response against the antigen contained in the immunogenic  
15 composition of the invention, comprising the step of administering to a host an effective amount of the above mentioned immunogenic compositions. Preferably this is also a method of inducing faster protection against the pathogen from which the antigen is derived.

Such a method would be extremely valuable for treating patients with  
20 compromised or weakened immune systems, such as the elderly (people over 55 years). Thus another embodiment is a method of protecting an elderly patient against a pathogen by administering to said patient an effective amount of the immunogenic composition mentioned above in which the antigen is derived from said pathogen.

Further aspects include a use of the above mentioned immunogenic  
25 preparations in the manufacture of a medicament for the treatment of a disease caused by the pathogen from which the antigen present within is derived. A use of blebs derived from *Moraxella catarrhalis* as an adjuvant in an immunogenic composition comprising one or more pneumococcal capsular polysaccharides, a use of blebs derived from *Moraxella catarrhalis* as an adjuvant in an immunogenic composition  
30 comprising one or more pneumococcal (or *H. influenzae*) protein antigens, a use of blebs derived from non-typeable *H. influenzae* as an adjuvant in an immunogenic composition comprising one or more pneumococcal capsular polysaccharides, and a

use of blebs derived from non-typeable *H. influenzae* as an adjuvant in an immunogenic composition comprising one or more pneumococcal (or *M. catarrhalis*) protein antigens, are further envisioned embodiments.

5 *Genetically-engineered bleb adjuvants*

The bleb adjuvant of the present invention may be improved using a general set of tools and methods for making genetically engineered blebs from Gram-negative bacterial strains. The invention includes methods used to make recombinant bleb adjuvants more immunogenic, less toxic and safer for their use in a human and/or  
10 animal vaccine. Moreover, the present invention also describes specific methods necessary for constructing, producing, obtaining and using recombinant, engineered blebs from various Gram-negative bacteria, for vaccine/adjuvant purposes. By the methods of the invention, the biochemical composition of bacterial blebs can be manipulated by acting upon/altering the expression of bacterial genes encoding  
15 products present in or associated with bacterial outer-membrane blebs (outer membrane proteins or OMPs). The production of blebs using a method of genetic modification to increase, decrease or render conditional the expression of one or more genes encoding outer-membrane components are also included in the scope of this invention.

20 For clarity, the term "expression cassette" will refer herein to all the genetic elements necessary to express a gene or an operon and to produce and target the corresponding protein(s) of interest to outer-membrane blebs, derived from a given bacterial host. A non-exhaustive list of these features includes control elements (transcriptional and/or translational), protein coding regions and targeting signals,  
25 with appropriate spacing between them. Reference to the insertion of promoter sequences means, for the purposes of this invention, the insertion of a sequence with at least a promoter function, and preferably one or more other genetic regulatory elements comprised within an expression cassette. Moreover, the term "integrative cassette" will refer herein to all the genetic elements required to integrate a DNA  
30 segment in given bacterial host. A non-exhaustive list of these features includes a delivery vehicle (or vector), with recombinogenic regions, and selectable and counter selectable markers.

Again for the purpose of clarity, the terms 'engineering a bacterial strain to produce less of said antigen' refers to any means to reduce the expression of an antigen of interest, relative to that of the non-modified (i.e., naturally occurring) bleb such that expression is at least 10% lower than that of the non-modified bleb. Preferably it is at least 50% lower. "Stronger promoter sequence" refers to a regulatory control element that increases transcription for a gene encoding antigen of interest. "Upregulating expression" refers to any means to enhance the expression of an antigen of interest, relative to that of the non-modified (i.e., naturally occurring) bleb. It is understood that the amount of 'upregulation' will vary depending on the particular antigen of interest but will not exceed an amount that will disrupt the membrane integrity of the bleb. Upregulation of an antigen refers to expression that is at least 10% higher than that of the non-modified bleb. Preferably it is at least 50% higher. More preferably it is at least 100% (2 fold) higher.

Aspects of the invention relate to individual methods for making improved engineered bleb adjuvants, to a combination of such methods, and to the bleb compositions made as a result of these methods. Another aspect of the invention relates to the genetic tools used in order to genetically modify a chosen bacterial strain in order to extract improved engineered blebs from said strain.

The engineering steps of the processes of the invention can be carried out in a variety of ways known to the skilled person. For instance, sequences (e.g. promoters or open reading frames) can be inserted, and promoters/genes can be disrupted by the technique of transposon insertion. For instance, for upregulating a gene's expression, a strong promoter could be inserted via a transposon up to 2 kb upstream of the gene's initiation codon (more preferably 200-600 bp upstream, most preferably approximately 400 bp upstream). Point mutation or deletion may also be used (particularly for down-regulating expression of a gene).

Such methods, however, may be quite unstable or uncertain, and therefore it is preferred that the engineering step [particularly for processes a), b), c), d), e), h) and i) as described below] is performed via a homologous recombination event. Preferably, the event takes place between a sequence (a recombinogenic region) of at least 30 nucleotides on the bacterial chromosome, and a sequence (a second recombinogenic region) of at least 30 nucleotides on a vector transformed within the strain. Preferably



the regions are 40-1000 nucleotides, more preferably 100-800 nucleotides, most preferably 500 nucleotides). These recombinogenic regions should be sufficiently similar that they are capable of hybridising to one another under highly stringent conditions (as defined later).

5           Recombination events may take place using a single recombinogenic region on chromosome and vector, or via a double cross-over event (with 2 regions on chromosome and vector). In order to perform a single recombination event, the vector should be a circular DNA molecule. In order to perform a double recombination event, the vector could be a circular or linear DNA molecule (see Figure 7). It is  
10   preferable that a double recombination event is employed and that the vector used is linear, as the modified bacterium so produced will be more stable in terms of reversion events. Preferably the two recombinogenic regions on the chromosome (and on the vector) are of similar (most preferably the same) length so as to promote double cross-overs. The double cross-over functions such that the two recombinogenic  
15   regions on the chromosome (separated by nucleotide sequence 'X') and the two recombinogenic regions on the vector (separated by nucleotide sequence 'Y') recombine to leave a chromosome unaltered except that X and Y have interchanged. The position of the recombinogenic regions can both be positioned upstream or downstream of, or may flank, an open reading frame of interest. These regions can consist  
20   of coding, non-coding, or a mixture of coding and non-coding sequence. The identity of X and Y will depend on the effect desired. X may be all or part of an open reading frame, and Y no nucleotides at all, which would result in sequence X being deleted from the chromosome. Alternatively Y may be a strong promoter region for insertion upstream of an open reading frame, and therefore X may be no nucleotides at all.

25           Suitable vectors will vary in composition depending what type of recombination event is to be performed, and what the ultimate purpose of the recombination event is. Integrative vectors used to deliver region Y can be conditionally replicative or suicide plasmids, bacteriophages, transposons or linear DNA fragments obtained by restriction hydrolysis or PCR amplification. Selection of  
30   the recombination event is selected by means of selectable genetic marker such as genes conferring resistance to antibiotics (for instance kanamycin, erythromycin, chloramphenicol, or gentamycin), genes conferring resistance to heavy metals and/or

toxic compounds or genes complementing auxotrophic mutations (for instance *pur*, *leu*, *met*, *aro*).

Process a) and f) – Down regulation/Removal of Variable and non-protective immunodominant antigens in bleb adjuvants

Many surface antigens are variable among bacterial strains and as a consequence are protective only against a limited set of closely related strains. An aspect of this invention covers the reduction in expression, or, preferably, the deletion of the gene(s) encoding variable surface protein(s) which results in a bacterial strain producing blebs which, when administered in a vaccine, have a stronger potential for cross-reactivity against various strains due to a higher influence exerted by conserved proteins (retained on the outer membranes) on the vaccinee's immune system. Examples of such variable antigens include: for *Neisseria* - pili (PilC) which undergoes antigenic variations, PorA, Opa, TbpB, FrpB; for *H. influenzae* - P2, P5, pilin, IgA1-protease; and for *Moraxella* - CopB, OMP106.

Other types of gene that could be down-regulated or switched off are genes which, *in vivo*, can easily be switched on (expressed) or off by the bacterium. As outer membrane proteins encoded by such genes are not always present on the bacteria, the presence of such proteins in the bleb preparations can also be detrimental to the effectiveness of the vaccine for the reasons stated above. A preferred example to down-regulate or delete is *Neisseria* Opc protein. Anti-Opc immunity induced by an Opc containing bleb vaccine would only have limited protective capacity as the infecting organism could easily become Opc<sup>-</sup>. *H. influenzae* HgpA and HgpB are other examples of such proteins.

In process a), these variable or non-protective genes are down-regulated in expression, or terminally switched off. This has the above-mentioned surprising advantage of concentrating the immune system on better antigens that are present in low amounts on the outer surface of blebs.

The strain can be engineered in this way by a number of strategies including transposon insertion to disrupt the coding region or promoter region of the gene, or point mutations or deletions to achieve a similar result. Homologous recombination may also be used to delete a gene from a chromosome (where sequence X comprises

part (preferably all) of the coding sequence of the gene of interest). It may additionally be used to change its strong promoter for a weaker (or no) promoter (where nucleotide sequence X comprises part (preferably all) of the promoter region of the gene, and nucleotide sequence Y comprises either a weaker promoter region [resulting in a decreased expression of the gene(s)/operon(s) of interest], or no promoter region). In this case it is preferable for the recombination event to occur within the region of the chromosome 1000 bp upstream of the gene of interest.

Alternatively, Y may confer a conditional transcriptional activity, resulting in a conditional expression of the gene(s)/operon(s) of interest (down-regulation). This is useful in the expression of molecules that are toxic to or not well supported by the bacterial host.

Most of the above-exemplified proteins are integral OMPs and their variability may be limited only to one or few of their surface exposed loops. Another aspect of this invention [process g)] covers the deletion of DNA regions coding for these surface exposed loops which leads to the expression of an integral OMP containing conserved surface exposed loops. Surface exposed loops of *H. influenzae* P2 and P5 are preferred examples of proteins that could be transformed into cross-reactive antigens by using such a method. Again, homologous recombination is a preferred method of performing this engineering process.

20

#### Process b) - Promoter delivery and modulation:

A further aspect of the invention relates to modifying the composition of bleb adjuvants by altering *in situ* the regulatory region controlling the expression of gene(s) and/or operon(s) of interest. This alteration may include partial or total replacement of the endogenous promoter controlling the expression of a gene of interest, with one conferring a distinct transcriptional activity. This distinct transcriptional activity may be conferred by variants (point mutations, deletions and/or insertions) of the endogenous control regions, by naturally occurring or modified heterologous promoters or by a combination of both. Such alterations will preferably confer a transcriptional activity stronger than the endogenous one (introduction of a strong promoter), resulting in an enhanced expression of the gene(s)/operon(s) of interest (up-regulation). Such a method is particularly useful for enhancing the production of

30

immunologically relevant Bleb components such as outer-membrane proteins and lipoproteins (preferably conserved OMPs, usually present in blebs at low concentrations).

Typical strong promoters that may be integrated in *Neisseria* are *porA* [SEQ ID NO: 24], *porB* [SEQ ID NO:26], *lgtF*, *Opa*, *p110*, *lst*, and *hpuAB*. PorA and PorB are preferred as constitutive, strong promoters. It has been established (Example 9) that the PorB promoter activity is contained in a fragment corresponding to nucleotides -1 to -250 upstream of the initiation codon of *porB*. In *Moraxella*, it is preferred to use the *ompH*, *ompG*, *ompE*, *OmpB1*, *ompB2*, *ompA*, *OMPCD* and *Omp106* promoters, and in *H. influenzae*, it is preferred to integrate the P2, P4, P1, P5 and P6 promoters.

Using the preferred double cross-over homologous recombination technology to introduce the promoter in the 1000 bp upstream region, promoters can be placed anywhere from 30-970 bp upstream of the initiation codon of the gene to be up-regulated. Although conventionally it is thought the promoter region should be relatively close to the open reading frame in order to obtain optimal expression of the gene, the present inventors have surprisingly found that placement of the promoter further away from the initiation codon results in large increases in expression levels. Thus it is preferred if the promoter is inserted 200-600 bp from the initiation codon of the gene, more preferably 300-500 bp, and most preferably approximately 400 bp from the initiation ATG.

#### Process c) - Bleb components produced conditionally

The expression of some genes coding for certain bleb components is carefully regulated. The production of the components is conditionally modulated and depends upon various metabolic and/or environmental signals. Such signals include, for example, iron-limitation, modulation of the redox potential, pH and temperature variations, nutritional changes. Some examples of bleb components known to be produced conditionally include iron-regulated outer-membrane proteins from *Neisseria* and *Moraxella* (for instance TbpB, LbpB), and substrate-inducible outer-membrane porins. The present invention covers the use of the genetic methods described previously (process a) or b)) to render constitutive the expression of such



molecules. In this way, the influence of environmental signal upon the expression of gene(s) of interest can be overcome by modifying/replacing the gene's corresponding control region so that it becomes constitutively active (for instance by deleting part [preferably all] or the repressive control sequence – e.g. the operator region), or inserting a constitutive strong promoter. For iron regulated genes the *fur* operator may be removed. Alternatively, process i) may be used to deliver an additional copy of the gene/operon of interest in the chromosome which is placed artificially under the control of a constitutive promoter.

#### 10 Processes d), and e) - Detoxification of LPS

The toxicity of bleb adjuvant preparations presents one of the largest problems in the use of blebs in vaccines. A further aspect of the invention relates to methods of genetically detoxifying the LPS present in Blebs. Lipid A is the primary component of LPS responsible for cell activation. Many mutations in genes involved in this pathway lead to essential phenotypes. However, mutations in the genes responsible for the terminal modifications steps lead to temperature-sensitive (*htrB*) or permissive (*msbB*) phenotypes. Mutations resulting in a decreased (or no) expression of these genes (or decreased or no activity of the product of these genes) result in altered toxic activity of lipid A. Indeed, the non-lauroylated (*htrB* mutant) or non-myristoylated (*msbB* mutant) lipid A are less toxic than the wild-type lipid A. Mutations in the lipid A 4'-kinase encoding gene (*lpxK*) also decreases the toxic activity of lipid A.

Process d) thus involves either the deletion of part (or preferably all) of one or more of the above open reading frames or promoters. Alternatively, the promoters could be replaced with weaker promoters, or the enzyme activity of the gene product may be significantly reduced by site specific mutagenesis. Preferably the homologous recombination techniques described above are used to carry out the process.

The sequences of the *htrB* and *msbB* genes from *Neisseria meningitidis* B, *Moraxella catarrhalis*, and *Haemophilus influenzae* are additionally provided for this purpose.

30 LPS toxic activity could also be altered by introducing mutations in genes/loci involved in polymyxin B resistance (such resistance has been correlated with addition of aminoarabinose on the 4' phosphate of lipid A). These genes/loci could be *pmrE*

that encodes a UDP-glucose dehydrogenase, or a region of antimicrobial peptide-resistance genes common to many enterobacteriaceae which could be involved in aminoarabinose synthesis and transfer. The gene *pmrF* that is present in this region encodes a dolicol-phosphate manosyl transferase (Gunn J.S., Kheng, B.L., Krueger J.,  
5 Kim K., Guo L., Hackett M., Miller S.I. 1998. *Mol. Microbiol.* 27: 1171-1182).

Mutations in the PhoP-PhoQ regulatory system, which is a phospho-relay two component regulatory system (f. i. PhoP constitutive phenotype, PhoP<sup>c</sup>), or low Mg<sup>++</sup> environmental or culture conditions (that activate the PhoP-PhoQ regulatory system) lead to the addition of aminoarabinose on the 4'-phosphate and 2-hydroxymyristate  
10 replacing myristate (hydroxylation of myristate). This modified lipid A displays reduced ability to stimulate E-selectin expression by human endothelial cells and TNF- $\alpha$  secretion from human monocytes.

Process e) involves the upregulation of these genes using a strategy as described above (strong promoters being incorporated, preferably using homologous  
15 recombination techniques to carry out the process).

Alternatively, rather than performing any such mutation, a polymyxin B resistant strain could be used as a bleb adjuvant production strain (in conjunction with one or more of the other processes of the invention), as blebs from such strains also have reduced LPS toxicity (for instance as shown for meningococcus - van der Ley, P,  
20 Hamstra, HJ, Kramer, M, Steeghs, L, Petrov, A and Poolman, JT. 1994. *In*: Proceedings of the ninth international pathogenic Neisseria conference. The Guildhall, Winchester, England).

As a further alternative (and further aspect of the invention) the inventors provide a method of detoxifying a Gram-negative bacterial strain comprising the step  
25 of culturing the strain in a growth medium containing 0.1mg-100g of aminoarabinose per litre medium, and the bleb adjuvant derived from such a strain.

As a further still alternative, synthetic peptides that mimic the binding activity of polymyxin B (described below) may be added to the Bleb preparation in order to reduce LPS toxic activity (Rustici, A, Velucchi, M, Faggioni, R, Sironi, M, Ghezzi, P,  
30 Quataert, S, Green, B and Porro M 1993. *Science* 259: 361-365; Velucchi, M, Rustici, A, Meazza, C, Villa, P, Ghezzi, P and Porro, M. 1997. *J. Endotox. Res.* 4:).

Process f) - Anchoring homologous or heterologous proteins to outer-membrane bleb adjuvants whilst reducing the toxicity of LPS

A further aspect of this invention covers the use of genetic sequences encoding polymyxin B peptides (or analogues thereof) as a means to target fusion proteins to the outer-membrane. Polymyxin B is a cyclic peptide composed of non tRNA-  
5 encoded amino acids (produced by Gram-positive actinomycetal organisms) that binds very strongly to the Lipid A part of LPS present in the outer-membrane. This binding decreases the intrinsic toxicity of LPS (endotoxin activity). Peptides mimicking the structure of Polymyxin B and composed of canonical (tRNA encoded)  
10 amino acids have been developed and also bind lipid A with a strong affinity. These peptides have been used for detoxifying LPS. One of these peptides known as SAEP-2 (Nterminus-Lys-Thr-Lys-Cys-Lys-Phe-Leu-Lys-Lys-Cys-Cterminus) was shown to be very promising in that respect (Molecular Mapping and detoxifying of the Lipid A binding site by synthetic peptides (1993). Rustici, A., Velucchi, M., Faggioni, R.,  
15 Sironi, M., Ghezzi, P., Quataert, S., Green, B. and M. Porro. *Science* 259, 361-365).

The present process f) of the invention provides an improvement of this use. It has been found that the use of DNA sequences coding for the SEAP-2 peptide (or derivatives thereof), fused genetically to a gene of interest (encoding for instance a T cell antigen or a protective antigen that is usually secreted such as a toxin, or a  
20 cytosolic or periplasmic protein) is a means for targeting the corresponding recombinant protein to the outer-membrane of a preferred bacterial host (whilst at the same time reducing the toxicity of the LPS).

This system is suitable for labile proteins which would not be directly exposed to the outside of the bleb. The bleb would therefore act as a delivery vehicle which  
25 would expose the protein to the immune system once the blebs had been engulfed by T-cells. Alternatively, the genetic fusion should also comprise a signal peptide or transmembrane domain such that the recombinant protein may cross the outer membrane for exposure to the host's immune system.

This targeting strategy might be of particular interest in the case of genes  
30 encoding proteins that are not normally targeted to the outer-membrane. This methodology also allows the isolation of recombinant blebs enriched in the protein of interest. Preferably, such a peptide targeting signal allows the enrichment of outer

membrane blebs in one or several proteins of interest, which are naturally not found in that given subcellular localization. A non exhaustive list of bacteria that can be used as a recipient host for such a production of recombinant blebs includes *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Moraxella catarrhalis*, *Haemophilus influenzae*,  
5 *Pseudomonas aeruginosa*, *Chlamydia trachomatis*, and *Chlamydia pneumoniae*.

Although it is preferred that the gene for the construct is engineered into the chromosome of the bacterium [using process i)], an alternative preferred embodiment is for SAEP-2-tagged recombinant proteins to be made independently, and attached at a later stage to a bleb preparation.

10 A further embodiment is the use of such constructs in a method of protein purification. The system could be used as part of an expression system for producing recombinant proteins in general. The SAEP-2 peptide tag can be used for affinity purification of the protein to which it is attached using a column containing immobilised lipid A molecules.

15

#### Process h) - Cross-reactive polysaccharides on bleb adjuvant

The isolation of bacterial outer-membrane blebs from encapsulated Gram-negative bacteria often results in the co-purification of capsular polysaccharide. In some cases, this "contaminant" material may prove useful since polysaccharide may  
20 enhance the immune response conferred by other bleb components. In other cases however, the presence of contaminating polysaccharide material in bacterial bleb preparations may prove detrimental to the use of the blebs in a vaccine. For instance, it has been shown at least in the case of *N. meningitidis* that the serogroup B capsular polysaccharide does not confer protective immunity and is susceptible to induce an  
25 adverse auto-immune response in humans. Such human-like epitopes may also be present on LPS/LOS within the blebs. Consequently, process h) of the invention is the engineering of the bacterial strain for bleb production such that it is free of human-like epitopes, particularly capsular polysaccharide. The blebs will then be suitable for use in humans. A particularly preferred example of such a bleb preparation is one from *N.*  
30 *meningitidis* serogroup B devoid of capsular polysaccharide.

This may be achieved by using modified bleb production strains in which the genes necessary for polysaccharide biosynthesis and/or export have been impaired.



Inactivation of the gene coding for polysaccharide biosynthesis or export can be achieved by mutating (point mutation, deletion or insertion) either the control region, the coding region or both (preferably using the homologous recombination techniques described above). Moreover, inactivation of capsular biosynthesis genes may also be achieved by antisense over-expression or transposon mutagenesis. A preferred method is the deletion of some or all of the *Neisseria meningitidis* *cps* genes required for polysaccharide biosynthesis and export. For this purpose, the replacement plasmid pMF121 (described in Frosh et al.1990, *Mol. Microbiol.* 4:1215-1218) can be used to deliver a mutation deleting the *cpsCAD* (+ *galE*) gene cluster. Alternatively the *siaD* gene could be deleted, or down-regulated in expression (the meningococcal *siaD* gene encodes alpha-2,3-sialyltransferase, an enzyme required for capsular polysaccharide and LOS synthesis). Such mutations may also remove host-similar structures on the saccharide portion of the LPS of the bacteria.

15 Process i) - Delivery of one or more further copies of a Gene and/or operon in a host chromosome, or delivery of a heterologous gene and/or operon in a host chromosome.

An efficient strategy to modulate the composition of a Bleb preparation is to deliver one or more copies of a DNA segment containing an expression cassette into the genome of a Gram-negative bacterium. A non exhaustive list of preferred bacterial species that could be used as a recipient for such a cassette includes *Neisseria meningitidis*, *Neisseiria gonorrhoeae*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Chlamydia trachomatis*, *Chlamydia pneumoniae*. The gene(s) contained in the expression cassette may be homologous (or endogenous) (i.e. exist naturally in the genome of the manipulated bacterium) or heterologous (i.e. do not exist naturally in the genome of the manipulated bacterium). The reintroduced expression cassette may consist of unmodified, "natural" promoter/gene/operon sequences or engineered expression cassettes in which the promoter region and/or the coding region or both have been altered. A non-exhaustive list of preferred promoters that could be used for expression includes the promoters *porA*, *porB*, *lbpB*, *tbpB*, *p110*, *lst*, *hpuAB* from *N. meningitidis* or *N. gonorrhoeae*, the promoters p2, p5, p4, *ompF*, p1, *ompH*, p6, *hin47* from *H. influenzae*, the promoters *ompH*, *ompG*, *ompCD*, *ompE*, *ompB1*, *ompB2*, *ompA* of *M. catarrhalis*, the



promoter  $\lambda$ pL, *lac*, *tac*, *araB* of *Escherichia coli* or promoters recognized specifically by bacteriophage RNA polymerase such as the *E. coli* bacteriophage T7. A non-exhaustive list of preferred genes that could be expressed in such a system includes *Neisseria* NspA, Omp85, PilQ, TbpA/B complex, Hsf, PldA, HasR; *Chlamydia* MOMP, HMWP; *Moraxella* OMP106, HasR, PilQ, OMP85, PldA; *Bordetella pertussis* FHA, PRN, PT.

In a preferred embodiment of the invention the expression cassette is delivered and integrated in the bacterial chromosome by means of homologous and/or site specific recombination. Integrative vectors used to deliver such genes and/or operons can be conditionally replicative or suicide plasmids, bacteriophages, transposons or linear DNA fragments obtained by restriction hydrolysis or PCR amplification. Integration is preferably targeted to chromosomal regions dispensable for growth *in vitro*. A non exhaustive list of preferred loci that can be used to target DNA integration includes the *porA*, *porB*, *opa*, *opc*, *rmp*, *omp26*, *lecA*, *cps*, *lgtB* genes of *Neisseria meningitidis* and *Neisseria gonorrhoeae*, the *P1*, *P5*, *hmw1/2*, *IgA-protease*, *fimE* genes of NTHi; the *lecA1*, *lecA2*, *omp106*, *uspA1*, *uspA2* genes of *Moraxella catarrhalis*. Alternatively, the expression cassette used to modulate the expression of bleb component(s) can be delivered into a bacterium of choice by means of episomal vectors such as circular/linear replicative plasmids, cosmids, phasmids, lysogenic bacteriophages or bacterial artificial chromosomes. Selection of the recombination event can be selected by means of selectable genetic marker such as genes conferring resistance to antibiotics (for instance kanamycin, erythromycin, chloramphenicol, or gentamycin), genes conferring resistance to heavy metals and/or toxic compounds or genes complementing auxotrophic mutations (for instance *pur*, *leu*, *met*, *aro*).

#### *Heterologous Genes - Expression of foreign proteins in outer-membrane blebs*

Outer-membrane bacterial blebs represent a very attractive system to produce, isolate and deliver recombinant proteins. A further aspect of this invention is in respect of the expression, production and targeting of foreign, heterologous proteins to the outer-membrane, and the use of the bacteria to produce recombinant blebs.

A preferred method of achieving this is via a process comprising the steps of: introducing a heterologous gene, optionally controlled by a strong promoter sequence, into the chromosome of a Gram-negative strain by homologous recombination. Blebs may be made from the resulting modified strain.

5 A non-exhaustive list of bacteria that can be used as a recipient host for production of recombinant blebs includes *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Chlamydia trachomatis*, *Chlamydia pneumoniae*. The gene expressed in such a system can be of viral, bacterial, fungal, parasitic or higher eukaryotic origin.

10 A preferred application of the invention includes a process for the expression of *Moraxella*, *Haemophilus* and/or *Pseudomonas* outer-membrane proteins (integral, polytopic and/or lipoproteins) in *Neisseria meningitidis* recombinant blebs. The preferable integration loci are stated above, and genes that are preferably introduced are those that provide protection against the bacterium from which they were isolated.

15 Preferred protective genes for each bacterium are described below.

Further preferred applications are: blebs produced from a modified *Haemophilus influenzae* strain where the heterologous gene is a protective OMP from *Moraxella catarrhalis*; and blebs produced from a modified *Moraxella catarrhalis* strain where the heterologous gene is a protective OMP from *Haemophilus influenzae* (preferred loci for gene insertion are given above, and preferred protective antigens are described below).

A particularly preferred application of this aspect is in the field of the prophylaxis or treatment of sexually-transmitted diseases (STDs). It is often difficult for practitioners to determine whether the principal cause of a STD is due to gonococcus or *Chlamydia trachomatis* infection. These two organisms are the main causes of salpingitis – a disease which can lead to sterility in the host. It would therefore be useful if a STD could be vaccinated against or treated with a combined vaccine effective against disease caused by both organisms. The Major Outer Membrane Protein (MOMP) of *C. trachomatis* has been shown to be the target of protective antibodies. However, the structural integrity of this integral membrane protein is important for inducing such antibodies. In addition, the epitopes recognised by these antibodies are variable and define more than 10 serovars. The previously

described aspect of this invention allows the proper folding of one or more membrane proteins within a bleb outer membrane preparation. The engineering of a gonococcal strain expressing multiple *C. trachomatis* MOMP serovars in the outer membrane, and the production of blebs therefrom, produces a single solution to the multiple problems of correctly folded membrane proteins, the presentation of sufficient MOMP serovars to protect against a wide spectrum of serovars, and the simultaneous prophylaxis/treatment of gonococcal infection (and consequently the non-requirement of practitioners to initially decide which organism is causing particular clinical symptoms – both organisms can be vaccinated against simultaneously thus allowing the treatment of the STD at a very early stage). Preferred loci for gene insertion in the gonococcal chromosome are give above. Other preferred, protective *C. trachomatis* genes that could be incorporated are HMWP, PmpG and those OMPs disclosed in WO 99/28475.

#### 15 *Targeting of heterologous proteins to outer-membrane blebs:*

The expression of some heterologous proteins in bacterial blebs may require the addition of outer-membrane targeting signal(s). The preferred method to solve this problem is by creating a genetic fusion between a heterologous gene and a gene coding for a resident OMP as a specific approach to target recombinant proteins to blebs. Most preferably, the heterologous gene is fused to the signal peptides sequences of such an OMP.

#### Neisserial bleb preparations

One or more of the following genes (encoding protective antigens) are preferred for upregulation via processes b) and/or i) when carried out on a Neisserial strain, including gonococcus, and meningococcus (particularly *N. meningitidis* B): NspA (WO 96/29412), Hsf-like (WO 99/31132), Hap (PCT/EP99/02766), PorA, PorB, OMP85 (WO 00/23595), PilQ (PCT/EP99/03603), PldA (PCT/EP99/06718), FrpB (WO 96/31618), TbpA (US 5,912,336), TbpB, FrpA/FrpC (WO 92/01460), LbpA/LbpB (PCT/EP98/05117), FhaB (WO 98/02547), HasR (PCT/EP99/05989), lipo02 (PCT/EP99/08315), Tbp2 (WO 99/57280), MltA (WO 99/57280), and ctrA

(PCT/EP00/00135). They are also preferred as genes which may be heterologously introduced into other Gram-negative bacteria.

One or more of the following genes are preferred for downregulation via process a): PorA, PorB, PilC, TbpA, TbpB, LbpA, LbpB, Opa, and Opc.

5 One or more of the following genes are preferred for downregulation via process d): htrB, msbB and lpxK (or homologues thereof).

One or more of the following genes are preferred for upregulation via process e): pmrA, pmrB, pmrE, and pmrF (or homologues thereof).

10 Preferred repressive control sequences for process c) are: the *fur* operator region (particularly for either or both of the TbpB or LbpB genes); and the DtxR operator region.

One or more of the following genes are preferred for downregulation via process h): galE, siaA, siaB, siaC, siaD, ctrA, ctrB, ctrC, and ctrD (or homologues thereof).

15

#### *Pseudomonas aeruginosa* bleb preparations

One or more of the following genes (encoding protective antigens) are preferred for upregulation via processes b) and/or i): PcrV, OprF, OprI. They are also preferred as genes which may be heterologously introduced into other Gram-negative  
20 bacteria.

#### *Moraxella catarrhalis* bleb preparations

One or more of the following genes (encoding protective antigens) are preferred for upregulation via processes b) and/or i): OMP106 (WO 97/41731 & WO  
25 96/34960), HasR (PCT/EP99/03824), PilQ (PCT/EP99/03823), OMP85 (PCT/EP00/01468), lipo06 (GB 9917977.2), lipo10 (GB 9918208.1), lipo11 (GB 9918302.2), lipo18 (GB 9918038.2), P6 (PCT/EP99/03038), ompCD, CopB (Helminen ME, et al (1993) Infect. Immun. 61:2003-2010), D15 (PCT/EP99/03822), Omp1A1 (PCT/EP99/06781), Hly3 (PCT/EP99/03257), LbpA and LbpB (WO  
30 98/55606), TbpA and TbpB (WO 97/13785 & WO 97/32980), OmpE, UspA1 and UspA2 (WO 93/03761), and Omp21. They are also preferred as genes which may be heterologously introduced into other Gram-negative bacteria.

One or more of the following genes are preferred for downregulation via process a): CopB, OMP106, OmpB1, TbpA, TbpB, LbpA, and LbpB.

One or more of the following genes are preferred for downregulation via process d): htrB, msbB and lpxK (or homologues thereof).

5 One or more of the following genes are preferred for upregulation via process e): pmrA, pmrB, pmrE, and pmrF (or homologues thereof).

One or more of the following genes are preferred for downregulation via process h) to remove any human-like epitopes from LPS: galE, siaA, siaB, siaC, siaD, ctrA, ctrB, ctrC, and ctrD (or homologues thereof).

10

#### Haemophilus influenzae bleb preparations

One or more of the following genes (encoding protective antigens) are preferred for upregulation via processes b) and/or i): D15 (WO 94/12641), P6 (EP 281673), TbpA, TbpB, P2, P5 (WO 94/26304), OMP26 (WO 97/01638), HMW1, 15 HMW2, HMW3, HMW4, Hia, Hsf, Hap, Hin47, and Hif (all genes in this operon should be upregulated in order to upregulate pilin). They are also preferred as genes which may be heterologously introduced into other Gram-negative bacteria.

One or more of the following genes are preferred for downregulation via process a): P2, P5, Hif, IgA1-protease, HgpA, HgpB, HMW1, HMW2, Hxu, TbpA, 20 and TbpB.

One or more of the following genes are preferred for downregulation via process d): htrB, msbB and lpxK (or homologues thereof).

One or more of the following genes are preferred for upregulation via process e): pmrA, pmrB, pmrE, and pmrF (or homologues thereof).

25 One or more of the following genes are preferred for downregulation via process h) to remove any human-like epitopes from LPS: galE, siaA, siaB, siaC, siaD, ctrA, ctrB, ctrC, and ctrD (or homologues thereof).

#### Vaccine Formulations

30 A preferred embodiment of the invention is the formulation of the bleb adjuvant preparations of the invention in a vaccine which may also comprise a pharmaceutically acceptable excipient.



The manufacture of bleb preparations from any of the aforementioned modified strains may be achieved by any of the methods well known to a skilled person. Preferably the methods disclosed in EP 301992, US 5,597,572, EP 11243 or US 4,271,147 are used. Most preferably, the method described in Example 8 is used.

5 Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds Powell M.F. & Newman M.J.) (1995) Plenum Press New York).

The bleb adjuvants of the present invention may be advantageously combined with further adjuvants in the vaccine formulation of the invention. Suitable further  
10 adjuvants include an aluminium salt such as aluminum hydroxide gel (alum) or aluminium phosphate, but may also be a salt of calcium (particularly calcium carbonate), iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

15 Suitable Th1 adjuvant systems that may be used in combination with bleb adjuvant include, Monophosphoryl lipid A, particularly 3-de-O-acylated monophosphoryl lipid A, and a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL) together with an aluminium salt. An enhanced system involves the combination of a monophosphoryl lipid A and a  
20 saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol as disclosed in WO96/33739. A particularly potent adjuvant formulation to be used with bleb adjuvant involves QS21 3D-MPL and tocopherol in an oil in water emulsion (described in WO95/17210) and is a preferred formulation.

25 The adjuvant may additionally comprise a saponin, more preferably QS21. It may also additionally comprise an oil in water emulsion and tocopherol. Unmethylated CpG containing oligo nucleotides (WO 96/02555) are also preferential inducers of a TH1 response and are suitable for use with bleb adjuvant in the present invention.

30 The vaccine preparations (bleb adjuvant mixed with antigen) of the present invention may be used to protect or treat a mammal susceptible to infection, by means of administering said vaccine via systemic or mucosal route. These administrations

may include injection *via* the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary, respiratory, genitourinary tracts. Thus one aspect of the present invention is a method of immunizing a human host against a disease caused by infection of a gram-negative bacteria, which method comprises administering to the host an immunoprotective dose of a protective antigen derived from said bacterium mixed with the bleb adjuvant of the present invention. The vaccine compositions of the present invention are particularly suitable for intranasal use. Further adjuvants such as Laureth-9 may also be included.

The amount of antigen in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees (as defined above).

#### Ghost or Killed Whole cell adjuvants

The inventors envisage that the above improvements to bleb adjuvants and resulting vaccine compositions can be easily extended to ghost or killed whole cell adjuvants preparations and vaccines (with identical advantages). The modified Gram-negative strains of the invention from which the bleb preparations are made can also be used to make ghost and killed whole cell adjuvant preparations. Methods of making ghost preparations (empty cells with intact envelopes) from Gram-negative strains are well known in the art (see for example WO 92/01791). Methods of killing whole cells to make inactivated cell preparations for use in vaccines are also well known. The terms 'bleb adjuvant preparations' and 'vaccines comprising bleb adjuvant' as well as the processes described throughout this document are therefore applicable to the terms 'ghost adjuvant preparation' and 'vaccines comprising ghost adjuvant', and 'killed whole cell adjuvant preparation' and 'vaccine comprising killed whole cell adjuvant', respectively, for the purposes of this invention.

#### Combinations of methods a) – i)

It may be appreciated that one or more of the above processes may be used to produce a modified strain from which to make improved bleb adjuvant preparations of the invention. Preferably one such process is used, more preferably two or more (2, 3,

4, 5, 6, 7, 8 or 9) of the processes are used in order to manufacture the bleb adjuvant. As each additional method is used in the manufacture of the adjuvant (particularly from processes d), e) and h)), each improvement works in conjunction with the other methods used in order to make an optimised engineered bleb adjuvant preparation.

5 A preferred meningococcal (particularly *N. meningitidis* B) bleb adjuvant preparation comprises the use of processes d) and h) and/or e). Such bleb preparations are safe (no structures similar to host structures), and non-toxic, but are still potent adjuvants. All the above elements work together in order to provide an optimised bleb adjuvant.

10 Similarly for *M. catarrhalis* and non-typeable *H. influenzae*, preferred bleb preparations comprise the use of processes d) and/or h) and/or e).

A further aspect of the invention is thus an safe and non-toxic Gram-negative bleb, ghost, or killed whole cell adjuvant suitable for paediatric use.

By paediatric use it is meant use in infants less than 4 years old.

15 By non-toxic it is meant that there is a significant (2-4 fold, preferably 10 fold) decrease of endotoxin activity as measured by the well-known LAL and pyrogenicity assays.

#### Nucleotide sequences of the invention

20 A further aspect of the invention relates to the provision of new nucleotide sequences which may be used in the processes of the invention. Specific upstream regions from various genes from various strains are provided which can be used in, for instance, processes a), b), d) and h). In addition, coding regions are provided for performing process d).

25

#### *General method for the analysis of the non-coding flanking region of a bacterial gene, and its exploitation for modulated expression of the gene in blebs*

The non-coding flanking regions of a specific gene contain regulatory elements important in the expression of the gene. This regulation takes place both at  
30 the transcriptional and translational level. The sequence of these regions, either upstream or downstream of the open reading frame of the gene, can be obtained by DNA sequencing. This sequence information allows the determination of potential

regulatory motifs such as the different promoter elements, terminator sequences, inducible sequence elements, repressors, elements responsible for phase variation, the Shine-Dalgarno sequence, regions with potential secondary structure involved in regulation, as well as other types of regulatory motifs or sequences.

5           This sequence information allows the modulation of the natural expression of the gene in question. The upregulation of the gene expression may be accomplished by altering the promoter, the Shine-Dalgarno sequence, potential repressor or operator elements, or any other elements involved. Likewise, downregulation of expression can be achieved by similar types of modifications. Alternatively, by changing phase  
10 variation sequences, the expression of the gene can be put under phase variation control, or may be uncoupled from this regulation. In another approach, the expression of the gene can be put under the control of one or more inducible elements allowing regulated expression. Examples of such regulation includes, but is not limited to, induction by temperature shift, addition of inductor substrates like selected  
15 carbohydrates or their derivatives, trace elements, vitamins, co-factors, metal ions, etc.

Such modifications as described above can be introduced by several different means. The modification of sequences involved in gene expression can be done *in vivo* by random mutagenesis followed by selection for the desired phenotype. Another approach consists in isolating the region of interest and modifying it by random  
20 mutagenesis, or site-directed replacement, insertion or deletion mutagenesis. The modified region can then be reintroduced into the bacterial genome by homologous recombination, and the effect on gene expression can be assessed. In another approach, the sequence knowledge of the region of interest can be used to replace or delete all or part of the natural regulatory sequences. In this case, the regulatory region  
25 targeted is isolated and modified so as to contain the regulatory elements from another gene, a combination of regulatory elements from different genes, a synthetic regulatory region, or any other regulatory region, or to delete selected parts of the wild-type regulatory sequences. These modified sequences can then be reintroduced into the bacterium via homologous recombination into the genome.

30           In process b), for example, the expression of a gene can be modulated by exchanging its promoter with a stronger promoter (through isolating the upstream sequence of the gene, *in vitro* modification of this sequence, and reintroduction into



the genome by homologous recombination). Upregulated expression can be obtained in both the bacterium as well as in the outer membrane vesicles shed (or made) from the bacterium.

In other preferred examples, the described approaches can be used to generate recombinant bacterial strains with improved characteristics for vaccine applications, as described above. These can be, but are not limited to, attenuated strains, strains with increased expression of selected antigens, strains with knock-outs (or decreased expression) of genes interfering with the immune response, and strains with modulated expression of immunodominant proteins.

10

SEQ ID NO:2-23, 25, 27-38 are all Neisserial upstream sequences (upstream of the initiation codon of various preferred genes) comprising approximately 1000 bp each. SEQ ID NO: 39-62 are all *M. catarrhalis* upstream sequences (upstream of the initiation codon of various preferred genes) comprising approximately 1000 bp each. SEQ ID NO: 63-75 are all *H. influenzae* upstream sequences (upstream of the initiation codon of various preferred genes) comprising approximately 1000 bp each. All of these can be used in genetic methods (particularly homologous recombination) for up-regulating, or down-regulating the open reading frames to which they are associated (as described before). SEQ ID NO: 76-81 are the coding regions for the HtrB and MsbB genes from Neisseria, *M. catarrhalis*, and *Haemophilus influenzae*. These can be used in genetic methods (particularly homologous recombination) for down-regulating (in particular deleting) part (preferably all) of these genes [process d)], or decreasing the activity of the gene product produced.

Another aspect of the invention is thus an isolated polynucleotide sequence which hybridises under highly stringent conditions to at least a 30 nucleotide portion of the nucleotides in SEQ ID NO: 2-23, 25, 27-81 or a complementary strand thereof. Preferably the isolated sequence should be long enough to perform homologous recombination with the chromosomal sequence if it is part of a suitable vector – namely at least 30 nucleotides (preferably at least 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, or 500 nucleotides). More preferably the isolated polynucleotide should comprise at least 30 nucleotides (preferably at least 40, 50, 60, 70, 80, 90, 100, 200,

30

300, 400, or 500 nucleotides) of SEQ ID NO: 2-23, 25, 27-81 or a complementary strand thereof.

As used herein, highly stringent hybridization conditions include, for example, 6X SSC, 5X Denhardt, 0.5% SDS, and 100 µg/mL fragmented and denatured salmon sperm DNA hybridized overnight at 65 °C and washed in 2X SSC, 0.1% SDS one  
5 time at room temperature for about 10 minutes followed by one time at 65 °C for about 15 minutes followed by at least one wash in 0.2X SSC, 0.1% SDS at room temperature for at least 3-5 minutes.

A further aspect is the use of the isolated polynucleotide sequences of the  
10 invention in performing a genetic engineering event (such as transposon insertion, or site specific mutation or deletion, but preferably a homologous recombination event) within 1000 bp upstream of a Gram-negative bacterial chromosomal gene in order to either increase or decrease expression of the gene. Preferably the strain in which the recombination event is to take place is the same as the strain from which the upstream  
15 sequences of the invention were obtained. However, the meningococcus A, B, C, Y and W and gonococcus genomes are sufficiently similar that upstream sequence from any of these strains may be suitable for designing vectors for performing such events in the other strains. This is may also be the case for *Haemophilus influenzae* and non-typeable *Haemophilus influenzae*.

## EXAMPLES

The examples below are carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. The examples are illustrative, but do not limit the invention. All references are  
5 incorporated by reference herein.

### Example 1: Construction of a *Neisseria meningitidis* serogroup B strain lacking capsular polysaccharides.

10 The plasmid pMF121 (Frosch et al., 1990) has been used to construct a *Neisseria meningitidis* B strain lacking the capsular polysaccharide. This plasmid contains the flanking regions of the gene locus coding for the biosynthesis pathway of the group B polysaccharide (B PS), and the erythromycin resistance gene. Deletion of the B PS resulted in loss of expression of the group B capsular polysaccharide as well as a  
15 deletion in the active copy of *galE* leading to the synthesis of galactose deficient LPS.

#### Strain transformation:

*Neisseria meningitidis* B H44/76 strain (B:15:P1.7, 16;Los 3,7,9) was selected for transformation. After an overnight CO<sub>2</sub> incubation on MH plate (without  
20 erythromycin), cells were collected in liquid MH containing 10 mM MgCl<sub>2</sub> (2 ml were used per MH plate) and diluted up to an OD of 0.1 (550 nm). To this 2 ml solution, 4 µl of the plasmid pMF121 stock solution (0.5 µg/ml) were added for a 6 hours incubation period at 37°C (with shaking). A control group was done with the same amount of *Neisseria meningitidis* B bacteria, but without addition of plasmid.  
25 After the incubation period, 100 µl of culture, as such, at 1/10, 1/100 and 1/1000 dilutions, were put in MH plates containing 5, 10, 20, 40 or 80 µg erythromycin /ml before incubation for 48 hours at 37°C.

#### Colony blotting:

30 After plate incubation, 20 colonies were grown and selected from the 10 and 20 µg erythromycin/ml MH plates, while there was no colony growth in the control group without plasmid transformation. The H44/76 wild type strain was unable to grow in

the selected erythromycin plates (10 to 80 µg erythromycin/ml). The day after, all the visible colonies were placed on new MH plates without erythromycin in order to let them grow. Afterwards, they were transferred onto nitrocellulose sheets (colony blotting) for presence of B polysaccharide. Briefly, colonies were blotted onto a nitrocellulose sheet and rinsed directly in PBS-0.05 % Tween 20 before cell inactivation for 1 hour at 56°C in PBS-0.05% Tween 20 (diluant buffer). Afterwards, the membrane was overlaid for one hour in the diluant buffer at room temperature (RT). Then, sheets were washed again for three times 5 minutes in the diluant buffer before incubation with the anti-B PS 735 Mab (Boehringer) diluted at 1/3000 in the diluant buffer for 2 hours at RT. After a new washing step (3 times 5 minutes), the monoclonal antibody was detected with a biotinylated anti-mouse Ig from Amersham (RPN 1001) diluted 500 times in the diluant buffer (one hour at RT) before the next washing step (as described above). Afterwards, sheets were incubated for one hour at RT with a solution of streptavidin-peroxidase complex diluted 1/1000 in the diluant buffer. After the last three washing steps using the same method, nitrocellulose sheets were incubated for 15 min in the dark using the revelation solution (30 mg of 4-chloro-1-naphtol solution in 10 ml methanol plus 40 ml PBS and 30 mcl of H<sub>2</sub>O<sub>2</sub> 37% from Merck). The reaction was stopped with a distilled water-washing step.

#### 20 Whole cell Elisas:

Whole cell Elisas were also done using the two transformed colonies ("D" and "R") and the wild type strain (H44/76) as coated bacteria (20 µg protein/ml), and a set of different monoclonal antibodies used to characterize *Neisseria meningitidis* strains. The following Mabs were tested: anti-B PS (735 from Dr Frosch), and the other Mabs from NIBSC: anti-B PS (Ref 95/750) anti-P1.7 (A-PorA, Ref 4025), anti-P1.16 (A-PorA, Ref 95/720), anti-Los 3,7,9 (A-LPS, Ref 4047), anti-Los 8 (A-LPS, Ref 4048), and anti-P1.2 (A-PorA Ref 95/696).

Microtiter plates (Maxisorp, Nunc) were coated with 100 µl of the recombinant meningococcal B cells solution overnight (ON) at 37°C at around 20 µg/ml in PBS. Afterwards, plates are washed three times with 300 µl of 150 mM NaCl - 0.05 % Tween 20, and were overlaid with 100 µl of PBS-0.3 % Casein and incubated for 30 min at room temperature with shaking. Plates were washed again



using the same procedure before incubation with antibodies. Monoclonal antibodies (100  $\mu$ l) were used at different dilutions (as shown in Figure 2) in PBS-0.3 % Casein 0.05 % Tween 20 and put onto the microplates before incubation at room temperature for 30 min with shaking, before the next identical washing step. 100  $\mu$ l of the anti-  
5 mouse Ig (from rabbit, Dakopatts E0413) conjugated to biotin and diluted at 1/2000 in PBS-0.3 % Casein - 0.05 % Tween 20 were added to the wells to detect bound monoclonal antibodies. After the washing step (as before), plates were incubated with a streptavidin-peroxidase complex solution (100  $\mu$ l of the Amersham RPN 1051) diluted at 1/4000 in the same working solution for 30 min at room temperature under  
10 shaking conditions. After this incubation and the last washing step, plates are incubated with 100  $\mu$ l of the chromogen solution (4 mg orthophenylenediamine (OPD) in 10 ml 0.1 M citrate buffer pH4.5 with 5  $\mu$ l H<sub>2</sub>O<sub>2</sub>) for 15 min in the dark. Plates are then read at 490/620 nm using a spectrophotometer.

## 15 Results:

Figure 1 shows that from the 20 isolated colonies, which were able to growth on the selected medium with erythromycin, only two (the "D" and the "R") colonies were shown negative for presence of B polysaccharide. Among the others, 16 were clearly positive for B PS and still resistant to erythromycin. This indicated that they  
20 integrated the plasmid into their genome, but in the wrong orientation, and keeping intact the B PS and LPS gene (no double crossing-over). Positive and negative controls were also tested on the plates, and showed that the H44/76 wild type NmB strain was clearly positive for the B polysaccharide, while meningococcus A (A1) and meningococcus C (C11) strains were clearly negative with this anti-B PS 735 Mab.  
25 These results indicate that around 10 % of the selected colonies correctly integrated the plasmid in their genome by making a double crossing-over, while the other strains/colonies were obtained after a simple crossing-over, keeping the B PS and LPS genes intact and expressed.

Using whole cell Elisa, results (Figure 2 and the Table below) clearly indicate  
30 that the two "D" and "R" transformants (derived from D and R colonies) can not be recognized anymore by the anti-B PS Mabs (735 and 95/750), nor by the anti-Los 3,7,9 and anti-Los 8 Mabs. However, when using specific anti-PorA Mabs, there is a

clear reaction with the anti-P1.7 and anti-P1.16 Mabs on the cells, as also observed in the wild-type strain. No reaction was observed with a non-specific anti-PorA Mab (anti-P1.2 mab). These results confirm that the PorA protein, and specifically P1.7 and P1.16 epitopes are still present after transformation, while B polysaccharide and  
 5 Los 3,7,9 and Los 8 epitopes (LPS) were not.

**Table :** Specificities of the monoclonal antibodies tested

Mabs Tested	Directed against	Result
Anti-B PS 735	B polysaccharide	++ on the wild type strain (-) on the "D" and "R" mutants
Anti-B PS 95/750 from NIBSC	B PS	++ on the wild type strain (-) on the "D" and "R" mutants
Anti-P1.7 (NIBSC)	Loop 1 of Porin A	++ on all wild type and mutants strains
Anti-P1.16 (NIBSC)	Loop 4 of Porin A	++ on all wild type and mutants strains
Anti-Los 3,7,9	LPS	++ on the wild type strain (-) on the "D" and "R" mutants
Anti-Los 8 (NIBSC)	LPS	+/- on the wild type strain (-) on the "D" and "R" mutants
Anti-P1.2 (NIBSC)	Anti-Porin A Sero-subtype 1.2	(-) on all wild type and mutants strains

10 **Example 2:** Construction of versatile gene delivery vectors (the pCMK series) targeting integration in the *porA* locus of *Neisseria meningitidis*.

A plasmid allowing homologous recombination and stable integration of foreign DNA in the *porA* locus of *Neisseria meningitidis* was constructed. This  
 15 delivery vector (genes, operons and/or expression cassettes) is useful for constructing *Neisseria meningitidis* strains producing recombinant, improved blebs. Typically, such a vector contains at least: (1) a plasmid backbone replicative in *E. coli* but not in *Neisseria meningitidis* (a suicide plasmid), (2) at least one, but preferably two regions

of homology for targeting the integration in a chromosomal locus such as *porA*, (3) Efficient transcriptional (promoter, regulatory region and terminator) and translational (optimised ribosome binding site and initiation codon) signals functional in *Neisseria meningitidis*, (4) a multiple cloning site and (5) selectable gene(s) allowing the  
5 maintenance of the plasmid in *E. coli* and the selection of integrants in *Neisseria meningitidis*. Additional elements include, for example, uptake sequences to facilitate the entry of foreign DNA in *Neisseria meningitidis*, and counter selectable markers such as *sacB*, *rpsL*, *gltS* to enhance the frequency of double cross-over events.

A schematic drawing of the vector constructed in this example and designated  
10 pCMK is represented in Figure 3. Its corresponding complete nucleotide sequence is shown in SEQ. ID NO:1. pCMK derives from a pSL1180 backbone (PharmaciaBiotech, Sweeden), a high copy-number plasmid replicative in *E. coli*, harbouring the *bla* gene (and thereby conferring resistance to ampicillin). In addition to this, pCMK functionally contains two *porA* flanking regions (*porA5'* and *porA3'*  
15 containing a transcription terminator) necessary for homologous recombination, a selectable marker conferring resistance to kanamycin, two uptake sequences, a *porA/lacO* chimeric promoter repressed in the *E.coli* host expressing *lacI<sup>q</sup>* but transcriptionally active in *Neisseria meningitidis*, and a multiple cloning site (5 sites present: *NdeI*, *KpnI*, *NheI*, *PmaI* and *SphI*) necessary for the insertion of foreign  
20 DNA in pCMK.

pCMK was constructed as follows. The *porA5'* and *porA3'* recombinogenic regions, the *porA/lacO* promoter were PCR amplified using the oligonucleotides listed in the table below, cloned in pTOPO and sequenced. These DNA fragments were successively excised from pTOPO and recloned in pSL1180. The kanamycin  
25 resistance cassette was excised from pUC4K (PharmaciaBiotech, Sweeden) and was introduced between the *porA5'* flanking region and the *porA/lacO* promoter region.

***Table: Oligonucleotides used in this work***

Oligonucleotides	Sequence	Remark(s)
PorA5' Fwd	5'-CCC AAG CTT <u>GCC GTC TGA</u> ATA CAT CCC GTC ATT CCT CA-3'	<i>Hind</i> III cloning site Uptake sequence ( )
PorA5' Rev	5'-CGA TGC TCG CGA CTC CAG AGA CCT CGT GCG GGC C-3'	<i>Nru</i> I cloning site
PorA3' Fwd	5'-GGA AGA TCT <u>GAT TAA ATA</u> GGC GAA AAT ACC AGC TAC GA-3'	<i>Bgl</i> II cloning site Stop codons ( )
PorA3' Rev	5'-GCC GAA TTC <u>TTC AGA CGG C</u> GC AGC AGG AAT TTA TCG G-3'	<i>Eco</i> RI cloning site Uptake sequence ( )
PoLa Rev1	5'- GAA TTG TTA TCC GCT CAC AAT TCC GGG CAA ACA CCC GAT AC-3'	
PoLa Rev2	5'-GAA TTC CAT ATG ATC GGC TTC CTT TTG TAA ATT TGA TAA AAA CCT AAA AAC ATC GAA TTG TTA TCC GCT C-3'	<i>Nde</i> I cloning site
PorAlacO Fwd	5'-AAG CTC TGC AGG AGG TCT GCG CTT GAA TTG-3'	<i>Pst</i> I cloning site
PorAlacO Rev	5'-CTT AAG GCA TAT GGG CTT CCT TTT GTA A- 3'	<i>Nde</i> I cloning site
PPA1	5'- GCG GCC GTT GCC GAT GTC AGC C-3'	
PPA2	5'-GGC ATA GCT GAT GCG TGG AAC TGC-3'	
N-full-01:	5'-GGG AAT TCC ATA TGA AAA AAG CAC TTG CCA CAC-3'	<i>Nde</i> I cloning site
Nde-NspA-3:	5'- GGA ATT CCA TAT GTC AGA ATT TGA CGC GCA C -3'	<i>Nde</i> I cloning site
PNS1	5'- CCG CGA ATT CGG AAC CGA ACA CGC CGT TCG-3'	<i>Eco</i> RI cloning site
PNS1	5'- CGT CTA GAC GTA GCG GTA TCC GGC TGC -3'	<i>Xba</i> I cloning site
PromD15-51X	5'- GGG CGA ATT CGC GGC CGC CGT CAA CGG CAC ACC CGT TG-3'	<i>Eco</i> RI and <i>Not</i> I cloning sites
PromD15-S2	5'- GCT CTA GAG CGG AAT GCG GTT TCA GAC G- 3'	<i>Xba</i> I cloning site
PNS4	5'- AGC TTT ATT TAA ATC CTT AAT TAA CGC GTC CGG AAA ATA TGC TTA TC_34	<i>Swa</i> I and <i>Pac</i> I cloning sites
PNS5	5'- AGC TTT GTT TAA ACC CTG TTC CGC TGC TTC GGC-3'	<i>Pme</i> I cloning site
D15-S4	5'- GTC CGC ATT TAA ATC CTT AAT TAA GCA GCC GGA CAG GGC GTG G-3'	<i>Swa</i> I and <i>Pac</i> I cloning sites
D15-S5	5'- AGC TTT GTT TAA AGG ATC AGG GTG TGG TCG GGC-3'	<i>Pme</i> I cloning site



**Example 3:** Construction of a *Neisseria meningitidis* serogroup B strain lacking both capsular polysaccharides and the major immunodominant antigen PorA.

Modulating the antigenic content of outer membrane blebs may be advantageous in improving their safety and efficacy in their use in vaccines, or diagnostic or therapeutic uses. Components such as the *Neisseria meningitidis* serogroup B capsular polysaccharides should be removed to exclude the risk of inducing autoimmunity (see example 1). Similarly, it is beneficial to suppress the immunodominance of major outer-membrane antigens such as PorA, which induce strain-specific bactericidal antibodies but fail to confer cross-protection. To illustrate such an approach, we used the pCMK(+) vector to construct a *Neisseria meningitidis* serogroup B strain lacking both capsular polysaccharides and the immunodominant PorA outer membrane protein antigen. For this purpose, a deletion of the *porA* gene was introduced in the H44/76 *cps*- strain, described in example 1 by means of homologous recombination.

The H44/76 *cps*- strain was prepared competent and transformed with two 2µg of supercoiled pCMK(+) plasmid DNA as described previously. Aliquot fractions of the transformation mixture (100µl) were plated on Mueller-Hinton plates supplemented with Kanamycin (200µg/ml) and incubated at 37°C for 24 to 48 hours. Kanamycin-resistant colonies were selected, restreaked on MH-Kn and grown for an additional 24 hours at 37°C. At that stage half of the bacterial culture was used to prepare glycerol stocks (15 % vol./vol.) and was kept frozen at -70°C. Another fraction (estimated to be 10<sup>8</sup> bacteria) was resuspended in 15 µl of distilled water, boiled ten minutes and used as a template for PCR screening. Two *porA* internal primers named , PPA1 and PPA2, were synthesized and used to perform PCR amplification on boiled bacterial lysates in the conditions described by the supplier (HiFi DNA polymerase, Boehringer Mannheim, GmbH). The thermal cycling used was the following: 25 times (94°C 1min., 52°C 1min., 72°C 3min.) and 1 time (72°C 10min., 4°C up to recovery). Since a double crossing-over between pCMK DNA and the chromosomal *porA* locus deletes the region required for #1 and #2 annealing, clones lacking a 1170bp PCR amplification fragment were selected as *porA* deletion mutants. These PCR results were further confirmed by analyzing in parallel, the

presence of PorA in the corresponding bacterial protein extracts. For that purpose, another aliquot of bacteria (estimated to be  $5 \cdot 10^8$  bacteria) was re-suspended in 50  $\mu$ l of PAGE-SDS buffer (SDS 5%, Glycerol 30%, Beta-mercaptoethanol 15%, Bromophenol blue 0.3mg/ml, Tris-HCl 250 mM pH6.8), boiled (100°C) frozen (-20°C) / boiled (100°C) three times and was separated by PAGE-SDS electrophoresis on a 12.5 % gel. Gels were then stained by Coomassie Brilliant blue R250 or transferred to a nitrocellulose membrane and probed with an anti-PorA monoclonal antibody as described in Maniatis *et al.* As represented in Figure 4, both Coomassie and immunoblot staining confirmed that *porA* PCR negative clones do not produce detectable levels of PorA. This result confirm that the pCMK vector is functional and can be used successfully to target DNA insertion in the *porA* gene, abolishing concomitantly the production of the PorA outer membrane protein antigen.

15 **Example 4:** Up-regulation of the NspA outer membrane protein production in blebs derived from a recombinant *Neisseria meningitidis* serogroup B strain lacking functional *porA* and *cps* genes.

Enriching bleb vesicles with protective antigens is advantageous for improving the efficiency and the coverage of outer membrane protein-based vaccines. In that context, recombinant *Neisseria meningitidis* strains lacking functional *cps* and *porA* genes were engineered so that the expressions level of the outer-membrane protein NspA was up-regulated. For that purpose, the gene coding for NspA was PCR amplified using the N01-full-*NdeI* and *NdeI*-3' oligonucleotide primers (see table in example 2). The conditions used for PCR amplification were those described by the supplier (HiFi DNA polymerase, Boehringer Mannheim, GmbH). Thermal cycling done was the following: 25 times (94°C 1min., 52°C 1min., 72°C 3min.) and 1 time (72°C 10min., 4°C up to recovery). The corresponding amplicon was digested with *NdeI* and inserted in the *NdeI* restriction site of the pCMK(+) delivery vector. Insert orientation was checked and recombinant plasmids, designed pCMK(+)-NspA, were purified at a large scale using the QIAGEN maxiprep kit and 2  $\mu$ g of this material was used to transform a *Neisseria meningitidis* serogroup B strain lacking functional *cps*

genes (strain described in example 1). Integration resulting from a double crossing-over between the pCMK(+)-NspA vector and the chromosomal *porA* locus were selected using a combination of PCR and Western blot screening procedures presented in example 3.

5 Bacteria (corresponding to about  $5 \cdot 10^8$  bacteria) were re-suspended in 50  $\mu$ l of PAGE-SDS buffer, frozen(-20°C) / boiled (100°C) three times and then were separated by PAGE-SDS electrophoresis on a 12.5 % gel. Gels were then stained by Coomassie Brilliant blue R250 or transferred to a nitrocellulose membrane and probed with an anti-NspA polyclonal serum. Both Coomassie (data not shown) and  
10 immunoblot staining (see figure 4) confirmed that *porA* PCR negative clones do not produce detectable levels of PorA. The expression of NspA was examined in Whole-cell bacterial lysates (WCBL) or outer-membrane bleb preparations derived from NmB [*cps*-, *porA*-] or NmB [*cps*-, *porA*-, *Nspa*+]. Although no difference was observable by Coomassie staining, immunoblotting with the anti-NspA polyclonal  
15 serum detected a 3-5 fold increased in the expression of NspA (with respect to the endogenous NspA level), both in WCBL and outer-membrane bleb preparations (see figure 5). This result confirm that the pCMK(+)-NspA vector is functional and can be used successfully to up-regulate the expression of outer membrane proteins such as NspA, abolishing concomitantly the production of the PorA outer membrane protein  
20 antigen.

**Example 5:** Up-regulation of the D15/Omp85 outer membrane protein antigen in blebs derived from a recombinant *Neisseria meningitidis* serogroup B strain lacking functional *cps* genes but expressing PorA.

25

Certain geographically isolated human populations (such as Cuba) are infected by a limited number of *Neisseria meningitidis* isolates belonging largely to one or few outer membrane protein serotypes. Since PorA is a major outer-membrane protein antigen inducing protective and strain-specific bactericidal antibodies, it is then  
30 possible to confer vaccine protection using a limited number of *porA* serotypes in a vaccine. In such a context, the presence of PorA in outer membrane vesicles may be advantageous, strengthening the vaccine efficacy of such recombinant improved

blebs. Such PorA containing vaccines, however, can be improved still further by increasing the level of other cross-reactive OMPs such as omp85/D15.

In the following example, the pCMK(+) vector was used to up-regulate the expression of the Omp85/D15 outer membrane protein antigen in a strain lacking functional *cps* genes but expressing *porA*. For that purpose, the gene coding for Omp85/D15 was PCR amplified using the D15-*NdeI* and D15-*NotI* oligonucleotide primers. The conditions used for PCR amplification were those described by the supplier (HiFi DNA polymerase, Boehringer Mannheim, GmbH). Thermal cycling done was the following: 25 times (94°C 1min., 52°C 1min., 72°C 3min.) and 1 time (72°C 10min., 4°C up to recovery). The corresponding amplicon was inserted in the pTOPO cloning vector according to the manufacturer's specifications and confirmatory sequencing was performed. This Omp85/D15 DNA fragment was excised from pTOPO by restriction hydrolysis using *NdeI/NsiI* and subsequently cloned in the corresponding restriction sites of the pCMK(+) delivery vector. Recombinant plasmids, designed pCMK(+)-D15 were purified on a large scale using the QIAGEN maxiprep kit and 2 µg of this material was used to transform a *Neisseria meningitidis* serogroup B strain lacking functional *cps* genes (strain described in example 1). In order to preserve the expression of *porA*, integration resulting from a single crossing-over (either in Omp85/D15 or in *porA*) were selected by a combination of PCR and Western blot screening procedures. Kanamycin resistant clones testing positive by *porA*-specific PCR and western blot were stored at -70°C as glycerol stocks and used for further studies.

Bacteria (corresponding to about  $5 \cdot 10^8$  bacteria) were re-suspended in 50 µl of PAGE-SDS buffer, frozen(-20°C) / boiled (100°C) three times and then were separated by PAGE-SDS electrophoresis on a 12.5 % gel. Gels were then stained by Coomassie Brilliant blue R250 or transferred to a nitrocellulose membrane and probed with an anti-*porA* monoclonal antibody. As represented in Figure 6, both Coomassie and immunoblot staining confirmed that *porA* PCR positive clones produce PorA.

The expression of D15 was examined using outer-membrane bleb preparations derived from NmB [*cps*-, *porA*-] or NmB [*cps*-, *porA*+, D15+]. Coomassie detected a significant increase in the expression of D15 (with respect to the endogenous D15 level), preparations (see Figure 6). This result confirmed that the pCMK(+)-D15



vector is functional and can be used successfully to up-regulate the expression of outer membrane proteins such as D15, without abolishing the production of the major PorA outer membrane protein antigen.

5 **Example 6: Construction of versatile promoter delivery vectors**

Rational: The rational of this approach is represented in Figure 7 and can be summarized in 7 essential steps. Some of these steps are illustrated below with the construction of Vector for up-regulating the expression of NspA and D15/Omp85.

10

Vector for up-regulating the expression of the NspA gene.

**Step 1.** A DNA region (997bp) located upstream from the NspA coding gene was discovered (SEQ. ID NO:2) in the private Incyte PathoSeq data base containing unfinished genomic DNA sequences of the *Neisseria meningitidis* strain ATCC  
15 13090. Two oligonucleotide primers referred to as PNS1 and PNS2 (see table in example 2) were designed using this sequence and synthesized. These primers were used for PCR amplification using genomic DNA extracted from the H44/76 strain.

**Step 2.** The corresponding amplicons were cleaned-up using the Wizard PCR kit (Promega, USA) and submitted to digestion with the *EcoRI/XbaI* restriction enzymes  
20 for 24 hours using the conditions described by the supplier (Boehringer Mannheim, Germany). The corresponding DNA fragments were gel purified and inserted in the corresponding sites of the pUC18 cloning vector. **Step 3.** Recombinant plasmids were prepared on a large scale and an aliquot fraction was used as a template for inverse PCR amplification. Inverse PCR was performed using the PNS4 and PNS5  
25 oligonucleotides using the following thermal cycling conditions: 25 times (94°C 1min., 50°C 1min., 72°C 3min.) and 1 time (72°C 10min., 4°C up to recovery). Linearized pUC 18 vectors harbouring a deletion in the NspA upstream region insert were obtained.

30 Vector for up-regulating the expression of the D15/omp85 gene.

**Step 1.** A DNA region (1000 bp) located upstream from the D15/omp85 coding gene was discovered (SEQ. ID NO:3) in the private Incyte PathoSeq database containing

unfinished genomic DNA sequences of the *Neisseria meningitidis* strain ATCC 13090. Two oligonucleotide primers referred to as PromD15-51X and PromD15-S2 (see table in example 2) were designed using this sequence and synthesized. These primers were used for PCR amplification using genomic DNA extracted from the H44/76 strain. **Step 2.** The corresponding amplicons were cleaned-up using the Wizard PCR kit (Promega, USA) and submitted to digestion with the *EcoRI/XbaI* restriction enzymes for 24 hours in the conditions described by the supplier (Boehringer Mannheim, Germany). The corresponding DNA fragments were gel purified and inserted in the corresponding sites of the pUC18 cloning vector. **Step 3.** Recombinant plasmids were prepared on a large scale and an aliquot fraction was used as a template for inverse PCR amplification. Inverse PCR was performed using the D15-S4 and D15-S5 oligonucleotides using the following thermal cycling conditions: 25 times (94°C 1min., 50°C 1min., 72°C 3min.) and 1 time (72°C 10min., 4°C up to recovery). Linearized pUC 18 vectors harbouring a deletion in the D15/omp85 upstream region insert were obtained.

**Example 7: Fermentation processes for producing recombinant blebs .**

The examples listed below describe methods for producing recombinant blebs lacking either capsular polysaccharides or capsular polysaccharides and PorA. Such a procedure may be used for a wide range of *Neisseria meningitidis* recombinant strains and may be adapted over an extended scale range.

Culture media: *Neisseria meningitidis* serogroup B strains were propagated in solid (FNE 004 AA, FNE 010 AA) or liquid (FNE 008 AA) culture media. These new media for growing meningococcus are advantageously free of animal products, and are considered a further aspect of the invention.

Components	FNE 004 AA	FNE 008 AA	FNE 010 AA
Agar	18 g/L	-	18 g/L
NaCl	6 g/L	6 g/L	6 g/L
Na-Glutamate	-	1.52 g/L	-
NaH <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O	2.2 g/L	2.2 g/L	2.2 g/L
KCl	0.09 g/L	0.09 g/L	0.09 g/L
NH <sub>4</sub> Cl	1.25 g/L	1.25 g/L	1.25 g/L
Glucose	5 g/L	20 g/L	5 g/L
Yeast Extract UF	-	2.5 g/L	-
Soy Pepton	5 g/L	30 g/L	5 g/L
CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.015 g/L	-	0.015 g/L
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.6 g/L	0.6 g/L	0.6 g/L
Erythromycine :	0.015 g/L	-	-
Kanamycine	-	-	0.2 g/L

5 Flask cultivation of *Neisseria meningitidis* serogroup B cps- recombinant blebs: This  
 was performed in two steps comprising preculture on solid medium followed by  
 liquid cultivation. **Solid pre-culture** A vial of seed was removed from freezer (-  
 80°C), thawed to room temperature and 0.1 mL was streaked into a Petri dish  
 containing 15 mL of FNE004AA (see above). The Petri dish was incubated at 37°C for  
 18 ± 2 hours. The surface growth was resuspended in 8 mL of FNE008AA (see  
 10 above) supplemented with 15 mg/L of erythromycin. **Flask culture.** 2 mL of  
 resuspended solid pre-culture were added to a 2 litre flask containing 400 mL of  
 FNE008AA supplemented with 15 mg/L of erythromycin. The flask was placed on a  
 shaking table (200 rpm) and incubated at 37°C for 16 ± 2 hours. The cells were  
 separated from the culture broth by centrifugation at 5000g at 4°C for 15 minutes.

15

Batch mode cultivation of *Neisseria meningitidis* serogroup B cps- recombinant  
blebs: This was performed in three steps comprising preculture on solid medium,  
 liquid cultivation and batch mode cultivation. **Solid pre-culture.** A vial of seed was  
 removed from freezer (-80°C), thawed to room temperature and 0.1 mL was streaked  
 20 into a Petri dish containing 15 mL of FNE004AA (see above). The Petri dish was

incubated at 37°C for 18 ± 2 hours. The surface growth was resuspended in 8 mL of FNE008AA (see above) supplemented with 15 mg/L of erythromycin. **Liquid pre-culture.** 2 mL of resuspended solid pre-culture were added to one 2 liters flask containing 400 mL of FNE008AA supplemented with 15 mg/L of erythromycin. The flask was placed on a shaking table (200 rpm) and incubated at 37°C for 16 ± 2 hours. The content of the flask was used to inoculate the 20 liters fermenter. **Batch mode culture in fermenter.** The inoculum (400 mL) was added to a pre-sterilized 20 liters (total volume) fermenter containing 10 L of FNE008AA supplemented with 15 mg/L of erythromycin. The pH was adjusted to and maintained at 7.0 by the automated addition of NaOH (25% w/v) and H<sub>3</sub>PO<sub>4</sub> (25% v/v). The temperature was regulated at 37°C. The aeration rate was maintained at 20 L of air / min and the dissolved oxygen concentration was maintained at 20% of saturation by the agitation speed control. The overpressure in the fermenter was maintained at 300 g/cm<sup>2</sup>. After 9 ± 1 hours, the culture was in stationary phase. The cells were separated from the culture broth by centrifugation at 5000g at 4°C for 15 minutes.

Flask cultivation of *Neisseria meningitidis* serogroup B cps-, PorA- recombinant blebs: This was performed in two steps comprising preculture on solid medium followed by liquid cultivation. **Solid pre-culture.** A vial of seed was removed from freezer (-80°C), thawed to room temperature and 0.1 mL was streaked into a Petri dish containing 15 mL of FNE010AA (see above). The Petri dish was incubated at 37°C for 18 ± 2 hours. The surface growth was resuspended in 8 mL of FNE008AA (see above) supplemented with 200 mg/L of kanamycin. **Flask culture.** 2 mL of resuspended solid pre-culture were added to a 2 litre flask containing 400 mL of FNE008AA supplemented with 200 mg/L of kanamycin. The flask was placed on a shaking table (200 rpm) and incubated at 37°C for 16 ± 2 hours. The cells were separated from the culture broth by centrifugation at 5000g at 4°C for 15 minutes.



**Example 8: Isolation and purification of blebs from meningococci devoid of capsular polysaccharide**

5 Recombinant blebs were purified as described below. The cell paste (42gr) was suspended in 211 ml of 0.1M Tris-Cl buffer pH 8.6 containing 10 mM EDTA and 0.5% Sodium Deoxycholate (DOC). The ratio of buffer to biomass was 5/1 (V/W). The biomass was extracted by magnetic stirring for 30 minutes at room temperature. Total extract was then centrifuged at 20,000g for 30 minutes at 4°C (13,000 rpm in a  
10 JA-20 rotor, Beckman J2-HS centrifuge). The pellet was discarded. The supernatant was ultracentrifuged at 125,000g for 2 hours at 4°C (40,000 rpm in a 50.2Ti rotor, Beckman L8-70M ultracentrifuge) in order to concentrate vesicles. The supernatant was discarded. The pellet was gently suspended in 25 ml of 50 mM Tris-Cl buffer pH 8.6 containing 2 mM EDTA, 1.2% DOC and 20% sucrose. After a second  
15 ultracentrifugation step at 125,000g for 2 hours at 4°C, vesicles were gently suspended in 44 ml of 3% sucrose and stored at 4°C. All solutions used for bleb extraction and purification contained 0.01% thiomersalate. As illustrated in Figure 8, this procedure yields protein preparations highly enriched in outer-membrane proteins such as PorA and PorB.

20

**Example 9: Identification of bacterial promoters suitable for up-regulation antigens-coding genes**

The use of strong bacterial promoter elements is essential to obtain up-regulation of genes coding for outer membrane proteins. In that context, we have  
25 shown previously that up-regulating the *Neisseria meningitidis nspA*, *hsf*, and *omp85* genes using the *porA* promoter has allowed us to isolate recombinant blebs enriched in the corresponding NspA, Hsf and Omp85 proteins. Alternatives to the *porA* promoter may be useful to obtain different levels of up-regulation, to overcome potential *porA* phase variation and/or to achieve conditional gene expression (iron-regulated promoters). Here we describe a method allowing the identification of a  
30 precise transcriptional start site of strong promoter elements likely to confer high level of expression in bacteria. Since promoter regulatory elements are classically

encompassed within 200 bp upstream and 50bp downstream from the +1 site (Collado-Vides J, Magasanik B, Gralla JD, 1991, *Microbiol Rev* 55(3):371-94), the result of such an experiment allows us to identify DNA fragments of about 250 bp carrying strong promoter activities. Major outer membrane proteins such as *Neisseria meningitidis* PorA, PorB & Rmp, *Haemophilus influenzae* P1, P2, P5 & P6, *Moraxella catarrhalis* OmpCD, OmpE, as well as some cytoplasmic and/or iron regulated proteins of these bacteria possess strong promoter elements. As a validation of this general methodology, we mapped the transcriptional start site of the strong *Neisseria meningitidis* *porA* and *porB* promoters using rapid amplification of cDNA elements (5' RACE).

The principles of 5' RACE are the following: 1) Total RNA extraction using QIAGEN "RNeasy" Kit. Genomic DNA removing by DNase treatment followed by QIAGEN purification; 2) mRNA reverse transcription with a *porA* specific 3' end primer (named porA3). Expected cDNA size: 307 nt. RNA removing by alkaline hydrolysis; 3) Ligation of a single-stranded DNA oligo anchor (named DT88) to the 3' end of the cDNA using T4 RNA ligase. Expected product size: 335 nt. Amplification of the anchor-ligated cDNA using a combination of hemi-nested PCR; 4) PCR amplification of the anchor-ligated cDNA using a complementary-sequence anchor primer as the 5' end primer (named DT89) and a 3'end primer (named p1-2) which is internal to the 3'end RT primer porA3. Expected product size: 292 bp; 5) PCR amplification of previous PCR products using DT89 as 5'end primer and p1-1 as 3'end primer (internal to p1-2). Expected product size: 211bp; and 6) Sequencing with p1-1 primer (expected products size can be calculated because *porA* transcription start site is known: 59 nt before the "ATG" translation start site).

### Experimental procedure

Total RNA was extracted from approximately  $10^9$  cells of *Neisseria meningitidis* serogroup B *cps- porA+* strain. Extraction of 1 ml of a liquid culture at appropriate optical density ( $OD_{600} = 1$ ) was performed by the QIAGEN "RNAeasy" kit according to the manufacturer's instructions. Chromosomal DNA was removed by addition of 10U of RNase-free DNase (Roche Diagnostics, Mannheim, Germany) to the 30  $\mu$ l of eluted RNA and was

incubated at 37°C for 15 min. The DNA-free RNA was purified with the same QIAGEN kit according to instructions.

Reverse transcription reactions were performed using primer porA3 and 200U of SUPERScript II reverse transcriptase (Life Technologies). The RT reactions were performed in  
5 a 50µl volume containing: 5µl of 2mM dNTP, 20 pmol of porA3 primer, 5µl of 10X SUPERScript II buffer, 9µl of 25mM MgCl<sub>2</sub>, 4µl of 0.1M DTT, 40U of recombinant ribonuclease inhibitor and 1 µg of total RNA. The porA3 primer was annealed stepwise (70°C for 2 min, 65°C for 1 min, 60°C for 1 min, 55°C for 1 min, 50°C for 1 min, and 45°C for 1 min) before the SUPERScript II was added. The RT reaction was performed at 42°C for  
10 30 min, followed by 5 cycles (50°C for 1 min, 53°C for 1 min and 56°C for 1 min) to destabilize RNA secondary structure. Two parallel reactions were performed with the reverse transcriptase omitted from one reaction as negative control.

The RNA was removed by alkaline hydrolysis cleavage with the addition of 1 µl of 0.5M EDTA followed by 12.5 µl of 0.2 M NaOH before incubation at 68°C for 5 min. The  
15 reactions were neutralized by adding 12.5 µl of 1 M Tris-HCl (pH7.4) and precipitated by the addition of 20 µg of glycogen (Roche Molecular Biochemicals, Mannheim, Germany), 5 µl of 3 M sodium acetate and 60 µl of isopropanol. Both samples were resuspended in 20 µl of 10:1 TE (10 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH8).

T4 RNA ligase was used to anchor a 5'-phosphorylated, 3'end ddCTP-blocked anchor  
20 oligonucleotide DT88 (see table below). Two parallel ligations were performed overnight at room temperature with each containing: 1.3 µl of 10X RNA ligase buffer (Roche Molecular Biochemicals), 0.4 µM DT88, 10 µl of either cDNA or RT control sample and 3 U of T4 RNA ligase. As negative controls, a second set of ligations reactions was performed, omitting the T4 RNA ligase. The resulting ligation-reaction mixtures were used directly without  
25 purification in the subsequent PCR.

The anchor-ligated cDNA was amplified using a combination of hemi-nested and hot-started PCR approaches to increase specificity and product yield. Four separate first-round PCR were performed on the RT/ligase reaction and controls in a 30 µl volume, each containing: 3 µl of 10X Taq Platinum buffer, 3µl of 25 mM MgCl<sub>2</sub>, 1 µl of 10mM dNTP, 10  
30 pmol of each primers and 1 µl of corresponding RNA ligation reaction. The PCR were hot started by the use of Taq Platinum (Life Technologies) DNA polymerase (2U added). The first ligation-anchored PCR (LA-PCR) was performed using 10 pmol of both the anchor-specific primer DT89 and the transcript-specific primer pl-2 (see table below) which is internal to the 3' end RT primer porA3. The PCR was performed using an initial 95°C for a 5  
35 min step (for DNA polymerase activation) followed by 10 cycles at 95°C for 10 s and 70°C

for 1 min (reducing one degree per cycle), 15 cycles at 95°C for 10 s and 60°C for 1 min. The second hemi-nested LA-PCR was performed under the same conditions using primer DT89 and the p1-2 internal primer, together with 10 pmol of p1-1 (see table below) and 1 µl of first-round PCR. Amplification products were purified using the QIAGEN "QIAquick PCR purification" kit according to manufacturer instructions before submitted to sequencing.

The CEQ™ Dye Terminator Cycle Sequencing kit (Beckman, France) was used to sequence the RACE PCR products using 10 pmol of primer p1-1. Sequencing reactions were performed according to the provided instructions and sequencing products were analyzed by the Ceq2000 DNA Analysis System (Beckman-Coulter).

DT88	5' GAAGAGAAGGTGGAAATGGCGTTTTGGC 3'
DT89	5' CCAAAACGCCATTTCACCTTCTCTTC 3'
porA3	5' CCAAATCCTCGCTCCCCTTAAAGCC 3'
p1-2	5' CGCTGATTTTCGTCCTGATGCGGC 3'
p1-1	5' GGTCAATTGCGCCTGGATGTTCTCTG 3'

#### Results for the *Neisseria meningitidis* *porA* promoter

The start of transcription for *Neisseria meningitidis* serogroup B (strain H44/76) *porA*-mRNA was mapped 59 bp upstream of the ATG start codon using the described 5'-RACE procedure. This result confirms the mapping performed by primer extension and published by van der Ende *et al* (1995). This result supports that a DNA fragment containing nucleotides -9 to -259 with regard to the *porA* ATG is suitable for driving strong gene expression in *Neisseria meningitidis* and possibly in other bacterial species such as *Haemophilus*, *Moraxella*, *Pseudomonas*.

#### Results for the *Neisseria meningitidis* *porB* promoter

The same experimental strategy has been applied for *Neisseria meningitidis* serogroup B (strain H44/76) *porB* transcription start site mapping. Primers listed in the table below correspond to 3' end RT primer (porB3), transcript-specific primer that is internal to the porB3 (porB2) and internal to the porB2 (porB1). porB3, porB2 and porB1 are respectively located 265 bp, 195 bp and 150 bp downstream the ATG start codon.

porB1	5' GGTAGCGGTTGTAAGTTCAGTAAGTTC 3'
porB2	5' GTCTTCTTGGCCTTTGAAGCCGATT 3'
porB3	5' GGAGTCAGTACCGGCGATAGATGCT 3'

Using porB1 and DT89 primers a ~200 bp PCR amplicon was obtained by performing 5' - RACE mapping. Since porB1 is located 150 bp from the *porB* ATG start



codon, this result supports that the *porB* transcriptional start site is located about 50 bp (+/- 30 bp) upstream of the *porB* ATG.

The exact nucleotide corresponding to transcription initiation is presently being determined by DNA sequencing. The above PCR result supports that a DNA fragment  
5 containing nucleotides -1 to -250 with regard to the *porB* ATG start codon is suitable for driving strong gene expression in *Neisseria meningitidis* and possibly in other bacterial species such as *Haemophilus*, *Moraxella*, *Pseudomonas*.

**Example 10:** Up-regulation of the *N. meningitidis* serogroup B *Omp85* gene by  
10 promoter replacement.

The aim of the experiment was to replace the endogenous promoter region of the D15/*Omp85* gene by the strong *porA* promoter in order to up-regulate the production of the D15/*Omp85* antigen. For that purpose, a promoter replacement plasmid was constructed using *E. coli* cloning methodologies. A DNA region (1000  
15 bp) located upstream from the *D15/omp85* coding gene was discovered (SEQ ID NO:3) in the private Incyte PathoSeq data base containing unfinished genomic DNA sequences of the *Neisseria meningitidis* strain ATCC 13090. The main steps of this procedure are represented in Figure 9. Briefly, a DNA fragment (1000bp) covering nucleotides -48 to -983 with respect to the *D15/Omp85* gene start codon (ATG) was  
20 PCR amplified using oligonucleotides **ProD15-51X** (5'-GGG CGA ATT CGC GGC CGC CGT CAA CGG CAC ACC GTT G-3') and **ProD15-52** (5'-GCT CTA GAG CGG AAT GCG GTT TCA GAC G-3') containing *EcoRI* and *XbaI* restriction sites (underlined) respectively. This fragment was submitted to restriction and inserted in pUC18 plasmid restricted with the same enzymes. The construct obtained was  
25 submitted to *in vitro* mutagenesis using the Genome Priming system (using the pGPS2 donor plasmid) commercialized by New England Biolabs (MA, USA). Clones having inserted a mini-transposon (derived from Tn7 and harboring a chloramphenicol resistance gene) were selected. One clone containing a mini-transposon insertion located in the *D15/Omp85* 5' flanking region, 401 bp  
30 downstream from the *EcoRI* site was isolated and used for further studies. This plasmid was submitted to circle PCR mutagenesis (Jones & Winistofer (1992), Biotechniques12: 528-534) in order to (i) delete a repeated DNA sequence (Tn7R) generated by the transposition process, (ii) insert meningococcal uptake sequences

required for transformation, and (iii) insert suitable restriction sites allowing cloning of foreign DNA material such as promoters. The circle PCR was performed using the **TnRD15-KpnI/XbaI** + US (5'-CGC CGG TAC CTC TAG AGC CGT CTG AAC CAC TCG TGG ACA ACC C-3') & **TnR03Cam(KpnI)** (5'-CGC CGG TAC CGC CGC TAA CTA TAA CGG TC-3') oligonucleotides containing uptake sequences and suitable restriction sites (*KpnI* and *XbaI*) underlined. The resulting PCR fragment was gel-purified, digested with *Asp718* (isoschizomer of *KpnI*) and ligated to a 184bp DNA fragment containing the *porA* promoter and generated by PCR using the **PorA-01** (5'-CGC CGG TAC CGA GGT CTG CGC TTG AAT TGT G-3') and **PorA02** (5'-CGC CGG TAC CTC TAG ACA TCG GGC AAA CAC CCG-3') oligonucleotides containing *KpnI* restriction sites. Recombinant clones carrying a *porA* promoter inserted in the correct orientation (transcription proceeding in the *EcoRI* to *XbaI* direction) were selected and used to transform a strain of *Neisseria meningitidis* serogroup B lacking capsular polysaccharide (*cps*-) and one of the major outer membrane proteins - PorA (*porA*-). Recombinant *Neisseria meningitidis* clones resulting from a double crossing over event (PCR screening using oligonucleotides **Cam-05** (5'-GTA CTG CGA TGA GTG GCA GG-3') & **proD15-52**) were selected on GC medium containing 5µg/ml chloramphenicol and analyzed for D15/Omp85 expression. As represented in Figure 10, the production of D15/Omp85 was significantly increased in the total protein extracts of *Nm* strains resulting from promoter replacement, when compared to parental strain (*cps*-). This result was also observed when analyzing outer-membrane blebs prepared from the same strains (see Figure 17). These results are attributable to the replacement of the endogenous *D15* promoter by the strong *porA* promoter. In addition, it was surprisingly found that expression, where the *porA* promoter was introduced approximately 400 bp upstream of the initiator codon, was approximately 50 times greater than when the promoter was placed approximately 100 bp upstream. Altogether, these experiments support that the promoter replacement strategy works and allows the up-regulation of the synthesis of integral outer-membrane proteins in outer-membrane blebs.

Certain geographically isolated human populations (such as Cuba) are infected by a limited number of *Neisseiria meningitidis* isolates belonging largely to one or

few outer membrane protein serotypes. Since PorA is a major outer-membrane protein antigen which can induce protective and strain-specific bactericidal antibodies, it may be possible to confer vaccine protection in such a population using a limited number of porA serotypes. Moreover, PorA may interact with or stabilize some other outer  
5 membrane proteins. In this context, the presence of PorA in outer membrane vesicles may be advantageous, strengthening the vaccine efficacy of such recombinant improved blebs.

For such a reason, it may be desirable to up-regulate the expression of D15/Omp85 outer membrane protein in a *Neisseria meningitidis* serogroup B strain  
10 lacking functional *cps* genes but expressing PorA. Genomic DNA was extracted from the recombinant *Neisseria meningitidis* serogroup B *cps*-, *porA*-, D15/Omp85+ strain using the QIAGEN Genomic Tips 100-G kit. 10µgr of this material was linearized and used to transform *Neisseria meningitidis* serogroup B *cps*- following a classical transformation protocol. Recombinant *Neisseria* were obtained on GC agar plates  
15 containing 5µgr/ml chloramphenicol.

Integrations resulting from a double crossing-over upstream of the *D15* gene were screened by PCR as described previously. As homologous recombinations can occur everywhere in the chromosome, a second PCR screening was performed to control the integrity of the *porA* locus in the recombinant strain. For this purpose,  
20 internal *porA* primers **PPA1** (5- GCG GCC GTT GCC GAT GTC AGC C -3') and **PpA2** ( 5- GGC ATA GCT GAT GCG TGG AAC TGC -3' ) were used in a PCR screening experiment. The amplification of an 1170bp fragment confirms the presence of the *porA* gene in the recombinant bacteria.

Recombinant bacteria (corresponding to about  $5 \cdot 10^8$  bacteria) can be re-  
25 suspended in 50 µl of PAGE-SDS buffer, frozen(-20°C) / boiled (100°C) three times and then separated by PAGE-SDS electrophoresis on a 12.5 % gel. Gels can then be stained by Coomassie Brilliant blue R250 or transferred to a nitrocellulose membrane and probed either with an anti-porA monoclonal antibody or with an anti-D15/Omp85 rabbit polyclonal antibody. Analysis of outer-membrane blebs prepared from the same  
30 strains can also be performed.

**Example 11: Up-regulation of the Hsf protein antigen in a recombinant *Neisseria meningitidis* serogroup B strain lacking functional *cps* genes but expressing PorA.**

As described above, in certain countries, the presence of PorA in outer membrane vesicles may be advantageous, and can strengthen the vaccine efficacy of recombinant improved blebs. In the following example, we have used a modified pCMK(+) vector to up-regulate the expression of the Hsf protein antigen in a strain lacking functional *cps* genes but expressing PorA. The original pCMK(+) vector contains a chimeric *porA/lacO* promoter repressed in *E. coli* host expressing *lacIq* but transcriptionally active in *Neisseria meningitidis*. In the modified pCMK(+), the native *porA* promoter was used to drive the transcription of the *hsf* gene. The gene coding for Hsf was PCR amplified using the HSF 01-*NdeI* and HSF 02-*NheI* oligonucleotide primers, presented in the table below. Because of the sequence of the HSF 01-*NdeI* primer the Hsf protein expressed will contain two methionine residues at the 5' end. The conditions used for PCR amplification were those described by the supplier (HiFi DNA polymerase, Boehringer Mannheim, GmbH). Thermal cycling was the following: 25 times (94°C 1min., 48°C 1min., 72°C 3min.) and 1 time (72°C 10min., 4°C up to recovery). The corresponding amplicon was subsequently cloned in the corresponding restriction sites of pCMK(+) delivery vector. In this recombinant plasmid, designed pCMK(+)-Hsf, we deleted the *lacO* present in the chimeric *porA/lacO* promoter by a recombinant PCR strategy (See Figure 12). The pCMK(+)-Hsf plasmid was used as a template to PCR amplify 2 separate DNA fragments:

-fragment 1 contains the *porA* 5' recombinogenic region, the Kanamycin resistance gene and the *porA* promoter. Oligonucleotide primers used, RP1(*SacII*) and RP2, are presented in the table below. RP1 primer is homologous to the sequence just upstream of the *lac* operator.

-fragment 2 contains the Shine-Dalgarno sequence from the *porA* gene, the *hsf* gene and the *porA* 3' recombinogenic region. Oligonucleotide primers used, RP3 and RP4(*ApaI*), are presented in the table below. RP3 primer is homologous to the sequence just downstream of the *lac* operator. The 3' end of fragment 1 and the 5' end of fragment 2 have 48 bases overlapping. 500ng of each PCR (1 and 2) were used for a final PCR reaction using primers RP1 and RP4. The final amplicon obtained was subcloned in pSL1180 vector restricted with *SacII* and *ApaI*. The modified plasmid



pCMK(+)-Hsf was purified at a large scale using the QIAGEN maxiprep kit and 2 µg of this material was used to transform a *Neisseria meningitidis* serogroup B strain lacking functional *cps* genes (the strain described in example 1). In order to preserve the expression of *porA*, integration resulting from a single crossing-over was selected by a combination of PCR and Western blot screening procedures. Kanamycin resistant clones testing positive by *porA*-specific PCR and western blot were stored at -70°C as glycerol stocks and used for further studies. Bacteria (corresponding to about 5.10<sup>8</sup> bacteria) were re-suspended in 50 µl of PAGE-SDS buffer, frozen (-20°C) / boiled (100°C) three times and then were separated by PAGE-SDS electrophoresis on a 12.5 % gel. The expression of Hsf was examined in Whole-cell bacterial lysates (WCBL) derived from *NmB* [Cps-, PorA+] or *NmB* [Cps-, PorA+, Hsf+]. Coomassie staining detected a significant increase in the expression of Hsf (with respect to the endogenous Hsf level) (See in Figure 13). This result confirms that the modified pCMK(+)-Hsf vector is functional and can be used successfully to up-regulate the expression of outer membrane proteins, without abolishing the production of the major PorA outer membrane protein antigen.

## 20 Oligonucleotides used in this work

Oligonucleotides	Sequence	Remark(s)
Hsf 01-Nde	5'- GGA ATT CCA TAT GAT GAA CAA AAT ATA CCG C-3'	<i>Nde</i> I cloning site
Hsf 02-Nhe	5'-GTA GCT AGC TAG CTT ACC ACT GAT AAC CGA C -3'	<i>Nhe</i> I cloning site
GFP-mut-Asn	5'-AAC TGC AGA ATT AAT ATG AAA GGA GAA GAA CTT TTC-3'	<i>Asn</i> I cloning site Compatible with <i>Nde</i> I
GFP-Spe	5'-GAC ATA CTA GTT TAT TTG TAG AGC TCA TCC ATG -3'	<i>Spe</i> I cloning site Compatible with <i>Nhe</i> I
RP1 (SacII)	5'- TCC CCG CGG GCC GTC TGA ATA CAT CCC GTC-3'	<i>Sac</i> II cloning site
RP2	5'-CAT ATG GGC TTC CTT TTG TAA ATT TGA GGG CAA ACA CCC GAT ACG TCT TCA-3'	



RP3	5'-AGA CGT ATC GGG TGT TTG CCC TCA AAT TTA CAA AAG GAA GCC CAT ATG -3'	
RP4(ApaI)	5'-GGG TAT TCC GGG CCC TTC AGA CGG CGC AGC AGG -3'	<i>ApaI</i> cloning site

**Example 12:** Expression of the Green Fluorescent Protein in a recombinant *Neisseria meningitidis* serogroup B strain lacking functional *cps* genes but expressing *PorA*.

5 In the following example, the pCMK vector was used to test the expression of a cytoplasmic heterologous protein in *Neisseria meningitidis*. The Green Fluorescent Protein was amplified from the pKen-Gfpmut2 plasmid with the primers GFP-Asn-mut2 and GFP-Spe (see table in Example 11). *AsnI* gives cohesive ends compatible with *NdeI*, *SpeI* gives cohesive ends compatible with *NheI*. The conditions used for  
10 PCR amplification were those described by the supplier (HiFi DNA polymerase, Boehringer Mannheim, GmbH). Thermal cycling was the following: 25 times (94°C 1min., 48°C 1min., 72°C 3min.) and 1 time (72°C 10min., 4°C up to recovery). The corresponding amplicon was subsequently cloned in the pCMK(+) delivery vector digested with *NdeI* and *NheI* restriction enzymes. In this recombinant plasmid,  
15 designed pCMK(+)-GFP, we deleted the *lacO* present in the chimeric *porA/lacO* promoter by a recombinant PCR strategy. The pCMK(+)-GFP plasmid was used as template to PCR amplify 2 separate DNA fragments:

-fragment 1 contained the *porA* 5' recombinogenic region, the Kanamycin resistance gene and the *porA* promoter. Oligonucleotide primers used, RP1(*SacII*) and  
20 RP2 (see table in example 11). RP1 primer is homologous to the sequence just upstream of the *lac* operator.

-fragment 2 contains the *PorA* Shine-Dalgarno sequence, the *gfp* gene and the *porA* 3' recombinogenic region. Oligonucleotide primers used, RP3 and RP4(*ApaI*), are presented in the table in example 11. RP3 primer is homologous to the sequence  
25 just downstream of the *lac* operator.

The 3'end of fragment 1 and the 5'end of fragment 2 have 48 bases overlapping. 500ng of each PCR (1 and 2) were used for a final PCR reaction using

primers RP1 and RP4. Twenty  $\mu\text{g}$  of this PCR fragment were used to transform a *Neisseria meningitidis* serogroup B strain lacking functional *cps* genes.

Transformation with linear DNA is less efficient than with circular plasmid DNA but all the recombinants obtained performed a double crossing-over (confirmed by a combination of PCR and Western blot screening procedures). Kanamycin resistant clones were stored at  $-70^{\circ}\text{C}$  as glycerol stocks and used for further studies. Bacteria (corresponding to about  $5 \cdot 10^8$  bacteria) were re-suspended in 50  $\mu\text{l}$  of PAGE-SDS buffer, frozen ( $-20^{\circ}\text{C}$ ) / boiled ( $100^{\circ}\text{C}$ ) three times and then were separated by PAGE-SDS electrophoresis on a 12.5 % gel.

The expression of GFP was examined in Whole-cell bacterial lysates (WCBL) derived from *NmB* [Cps-, PorA+] or *NmB* [Cps-, PorA-, GFP+]. Coomassie staining detected an expression of GFP absent in the recipient *Neisseria meningitidis* strain (see figure 14).

### Example 13: Up-regulation of the *N. meningitidis* serogroup B *NspA* gene by promoter replacement

The aim of the experiment was to replace the endogenous promoter region of the *NspA* gene by the strong *porA* promoter, in order to up-regulate the production of the NspA antigen. For that purpose, a promoter replacement plasmid was constructed using *E. coli* cloning methodologies. A DNA region (924bp) located upstream from the *NspA* coding gene was discovered (SEQ ID NO: 7) in the private Incyte PathoSeq data base containing unfinished genomic DNA sequences of the *Neisseria meningitidis* strain ATCC 13090. A DNA fragment (675bp) covering nucleotides -115 to -790 with respect to the *NspA* gene start codon (ATG) was PCR amplified using oligonucleotides PNS1' (5'-CCG CGA ATT CGA CGA AGC CGC CCT CGA C-3') and PNS2 (5'-CGT CTA GAC GTA GCG GTA TCC GGC TGC -3') containing *EcoRI* and *XbaI* restriction sites (underlined) respectively. The PCR fragment was submitted to restriction with *EcoRI* and *XbaI* and inserted in pUC18. This plasmid was submitted to circle PCR mutagenesis (Jones & Winistofer (1992), Biotechniques12: 528-534) in order to insert meningococcal uptake sequences required for transformation, and suitable restriction sites allowing cloning of a *CmR*/PorA promoter cassette. The circle PCR was performed using the

**BAD01-2** (5'- GGC GCC CGG GCT CGA GCT TAT CGA TGG AAA ACG CAG C-3') & **BAD02-2** (5'-GGC GCC CGG GCT CGA GTT CAG ACG GCG CGC TTA TAT AGT GGA TTA AC -3') oligonucleotides containing uptake sequences and suitable restriction sites (*Xma*I and *Xho*I) underlined. The resulting PCR fragment was gel-purified and digested with *Xho*I. The *CmR*/PorA promoter cassette was amplified from the pUC D15/Omp85 plasmid previously described, using primers

**BAD 15-2** (5'-GGC GCC CGG GCT CGA GTC TAG ACA TCG GGC AAA CAC CCG-3') & **BAD 03-2** (5'- GGC GCC CGG GCT CGA GCA CTA GTA TTA CCC TGT TAT CCC-3') oligonucleotides containing suitable restriction sites (*Xma*I, *Xba*I, *Spe*I and *Xho*I) underlined. The PCR fragment obtained was submitted to digestion and inserted in the circle PCR plasmid restricted with the corresponding enzymes. 10 µg of the recombinant plasmid were linearized and used to transform a strain of *Neisseria meningitidis* serogroup B lacking capsular polysaccharide (*cps*-) and one of the major outer membrane proteins - PorA (*porA*-). Recombinant *Neisseria meningitidis* clones resulting from a double crossing over event (PCR screening using oligonucleotides **BAD 25** (5'-GAG CGA AGC CGT CGA ACG C -3') & **BAD08** (5'-CTT AAG CGT CGG ACA TTT CC-3')) were selected on GC agar plates containing 5µg/ml chloramphenicol and analyzed for NspA expression. Recombinant bacteria (corresponding to about  $5 \cdot 10^8$  bacteria) were re-suspended in 50 µl of PAGE-SDS buffer, frozen (-20°C) / boiled (100°C) three times and then were separated by PAGE-SDS electrophoresis on a 12.5 % gel. Gels were then stained by Coomassie Brilliant blue R250 or transferred to a nitrocellulose membrane and probed either with an anti-PorA monoclonal antibody or with anti-NspA polyclonal antibody (figure 17). As for Omp85, there is a surprising indication that insertion of the promoter approximately 400 bp upstream of the NspA initiation codon expresses more protein than if placed approximately 100 bp upstream.

The same recombinant pUC plasmid can be used to up-regulate the expression of NspA in a *Neisseria meningitidis* serogroup B strain lacking functional *cps* gene but still expressing PorA.

**Example 14:** Up-regulation of the *N. meningitidis* serogroup B *pldA* (*omplA*) gene by promoter replacement.

The aim of the experiment was to replace the endogenous promoter region of the *pldA* (*omplA*) gene by the strong *porA* promoter in order to up-regulate the production of the PldA (OmplA1) antigen. For that purpose, a promoter replacement plasmid was constructed using *E. coli* cloning methodologies. A DNA region (373bp) located upstream from the *pldA* coding sequence was discovered (SEQ ID NO: 18) in the private Incyte PathoSeq data base of the *Neisseria meningitidis* strain ATCC 13090. This DNA contains the sequence coding for a putative *rpsT* gene. The stop codon of *rpsT* is located 169bp upstream the *pldA* ATG. To avoid the disruption of this potentially important gene, we decided to insert the *CmR*/PorA promoter cassette just upstream of the ATG of *pldA*. For that purpose, a DNA fragment of 992 bp corresponding to the the *rpsT* gene, the 169 bp intergenic sequence and the 499 first nucleotides of *pldA* gene was PCR amplified from *Neisseria meningitidis* serogroup B genomic DNA using oligonucleotides **PLA1 Amo5** (5'-GCC GTC TGA ATT TAA AAT TGC GCG TTT ACA G-3') and **PLA1 Amo3** (5'-GTA GTC TAG ATT CAG ACG GCG CAA TTT GGT TTC CGC AC -3') containing uptake sequences (underlined). PLA1 Amo3 contains also a *XbaI* restriction site. This PCR fragment was cleaned with a High Pure Kit (Roche, Mannheim, Germany) and directly cloned in a pGemT vector (Promega, USA). This plasmid was submitted to circle PCR mutagenesis (Jones & Winistofer (1992)) in order to insert suitable restriction sites allowing cloning of a *CmR*/PorA promoter cassette. The circle PCR was performed using the **CIRC1-Bgl** (5'CCT AGA TCT CTC CGC CCC CCA TTG TCG -3') & either **CIRC1-XH-RBS/2** (5'-CCG CTC GAG TAC AAA AGG AAG CCG ATA TGA ATA TAC GGA ATA TGC G-3') or **CIRC2-XHO/2** (5'-CCG CTC GAG ATG AAT ATA CGG AAT -3') oligonucleotides containing suitable restriction sites (*Bgl*III and *Xho*I) underlined. The *CmR*/PorA promoter cassette was amplified from the pUC D15/Omp85 plasmid previously described, using primers **BAD20** (5'- TCC CCC GGG AGA TCT CAC TAG TAT TAC CCT GTT ATC CC-3') and **CM-PORA-3** (5'- CCG CTC GAG ATA AAA ACC TAA AAA CAT CGG GC-3') containing suitable restriction sites (*Bgl*III and *Xho*I) underlined. This PCR fragment was cloned



in the circle PCR plasmid obtained with primers CIRC1-Bgl and CIRC1-XH-RBS/2. This plasmid can be used to transform *Neisseria meningitidis* serogroup B (*cps*-) and (*cps*- *porA*-) strains. Integration by double crossing-over in the upstream region of *pldA* will direct the insertion of the *porA* promoter directly upstream of the *pldA* ATG.

5 Another cassette was amplified from the genomic DNA of the recombinant *Neisseria meningitidis* serogroup B (*cps*-, *porA*-, D15/Omp85+) over-expressing D15/Omp85 by promoter replacement. This cassette contains the *cmR* gene, the *porA* promoter and 400bp corresponding to the 5' flanking sequence of the *D15/Omp85* gene. This sequence has been proven to be efficacious for up-regulation of the expression of  
 10 D15/Omp85 in *Neisseria* and will be tested for the up-regulation of the expression of other *Neisseria* antigens. Primers used for the amplification were **BAD 20** and **CM-PORA-D15/3** (5'- CGG CTC GAG TGT CAG TTC CTT GTG GTG C-3') containing *XhoI* restriction sites (underlined). This PCR fragment was cloned in the circle PCR plasmid obtained with primers CIRC1-Bgl and CIRC2-XHO/2. This  
 15 plasmid will be used to transform *Neisseria meningitidis* serogroup B (*cps*-) and (*cps*-, *porA*-) strains. Integration by double crossing-over in the upstream region of *pldA* will direct the insertion of the *porA* promoter 400bp upstream the *pldA* ATG.

**Example 15:** Up-regulation of the *N. meningitidis* serogroup B *tbpA* gene by  
 20 promoter replacement.

The aim of the experiment was to replace the endogenous promoter region of the *tbpA* gene by the strong *porA* promoter, in order to up-regulate the production of the TbpA antigen. For that purpose, a promoter replacement plasmid was constructed using *E. coli* cloning methodologies. A DNA region (731bp) located upstream from  
 25 the *tbpA* coding sequence was discovered (SEQ ID NO: 17) in the private Incyte PathoSeq data base of the *Neisseria meningitidis* strain ATCC 13090. This DNA contains the sequence coding for TbpB antigen. The genes are organized in an operon. The *tbpB* gene will be deleted and replaced by the *CmR/porA* promoter cassette. For that purpose, a DNA fragment of 3218bp corresponding to the 509bp 5' flanking  
 30 region of *tbpB* gene, the 2139bp *tbpB* coding sequence, the 87bp intergenic sequence and the 483 first nucleotides of *tbpA* coding sequence was PCR amplified from *Neisseria meningitidis* serogroup B genomic DNA using oligonucleotides **BAD16** (5'-



GGC CTA GCT AGC CGT CTG AAG CGA TTA GAG TTT CAA AAT TTA TTC-3') and **BAD17** (5'-GGC CAA GCT TCA GAC GGC GTT CGA CCG AGT TTG AGC CTT TGC-3') containing uptake sequences and *NheI* and *HindIII* restriction sites (underlined). This PCR fragment was cleaned with a High Pure Kit (Boehringer Mannheim, Germany) and directly cloned in a pGemT vector (Promega, USA). This plasmid was submitted to circle PCR mutagenesis (Jones & Winistofer (1992)) in order to (i) insert suitable restriction sites allowing cloning of a *CmR*/*PorA* promoter cassette and (ii) to delete 209bp of the 5' flanking sequence of *tbpB* and the *tbpB* coding sequence. The circle PCR was performed using the **BAD 18** (5'-TCC CCC GGG AAG ATC TGG ACG AAA AAT CTC AAG AAA CCG-3') & the **BAD 19** (5'-GGA AGA TCT CCG CTC GAG CAA ATT TAC AAA AGG AAG CCG ATA TGC AAC AGC AAC ATT TGT TCC G -3') oligonucleotides containing suitable restriction sites *XmaI*, *BglIII* and *XhoI* (underlined). The *CmR*/*PorA* promoter cassette was amplified from the pUC D15/*Omp85* plasmid previously described, using primers **BAD21** (5'- GGA AGA TCT CCG CTC GAG ACA TCG GGC AAA CAC CCG-3') & **BAD20** (5'- TCC CCC GGG AGA TCT CAC TAG TAT TAC CCT GTT ATC CC-3') containing suitable restriction sites *XmaI*, *SpeI*, *BglIII* and *XhoI* (underlined). This PCR fragment was cloned in the circle PCR plasmid. This plasmid will be used to transform *Neisseria meningitidis* serogroup B [*cps*-] and [*cps*- *porA*-] strains. Integration by double crossing-over in the upstream region of *tbpA* will direct the insertion of the *porA* promoter directly upstream of the *tbpA* ATG.

**Example 16: Up-regulation of the *N. meningitidis* serogroup B *pilQ* gene by promoter replacement.**

The aim of the experiment was to replace the endogenous promoter region of the *pilQ* gene by the strong *porA* promoter, in order to up-regulate the production of the PilQ antigen. For that purpose, a promoter replacement plasmid was constructed using *E. coli* cloning methodologies. A DNA region (772bp) located upstream from the *pilQ* coding gene was discovered (SEQ ID NO: 12) in the private Incyte PathoSeq data base of the *Neisseria meningitidis* strain ATCC 13090. This DNA contains the sequence coding for PilP antigen. The *pilQ* gene is part of an operon we do not want to disturb, pilins being essential elements of the bacteria. The *CmR*/*porA* promoter

cassette was introduced upstream the *pilQ* gene following the same strategy described for the up-regulation of the expression of the *pldA* gene. For that purpose, a DNA fragment of 866 bp corresponding to the 3' part of the *pilP* coding sequence, the 18bp intergenic sequence and the 392 first nucleotides of *pilQ* gene was PCR amplified from *Neisseria* serogroup B genomic DNA using **PQ-rec5-Nhe** (5'-CTA GCT AGC GCC GTC TGA ACG ACG CGA AGC CAA AGC-3') and **PQ-rec3-Hin** (GCC AAG CTT TTC AGA CGG CAC GGT ATC GTC CGA TTC G-3') oligonucleotides containing uptake sequences and *NheI* and *HindIII* restriction sites (underlined). This PCR fragment was directly cloned in a pGemT vector (Promega, USA). This plasmid was submitted to circle PCR mutagenesis (Jones & Winistofer (1992)) in order to insert suitable restriction sites allowing cloning of a *CmR*/PorA promoter cassette. The circle PCR was performed using the **CIRC1-PQ-Bgl** (5'-GGA AGA TCT AAT GGA GTA ATC CTC TTC TTA-3') & either **CIRC1-PQ-XHO** (5'-CCG CTC GAG TAC AAA AGG AAG CCG ATA TGA TTA CCA AAC TGA CAA AAA TC-3') or **CIRC2-PQ-X** (5'-CCG CTC GAG ATG AAT ACC AAA CTG ACA AAA ATC -3') oligonucleotides containing suitable restriction sites *BglIII* and *XhoI* (underlined). The *CmR*/PorA promoter cassette was amplified from the pUC D15/Omp85 plasmid previously described, using primers **BAD20** (5'- TCC CCC GGG AGA TCT CAC TAG TAT TAC CCT GTT ATC CC-3') and **CM-PORA-3** (5'- CCG CTC GAG ATA AAA ACC TAA AAA CAT CGG GCA AAC ACC C-3') containing suitable restriction sites *BglIII* and *XhoI* (underlined). This PCR fragment was cloned in the circle PCR plasmid obtained with primers CIRC1-PQ-Bgl and CIRC1-PQ-XHO. This plasmid can be used to transform *Neisseria meningitidis* serogroup B [*cps*-] and [*cps*-, *porA*-] strains. Integration by double crossing-over in the upstream region of *pilQ* will direct the insertion of the *porA* promoter directly upstream of the *pilQ* ATG.

Another cassette was amplified from the genomic DNA of the recombinant *Neisseria meningitidis* serogroup B [*cps*-, *porA*-, *D15/Omp85*+] over-expressing *D15/Omp85* by promoter replacement. This cassette contains the *cmR* gene, the *porA* promoter and 400bp corresponding to the 5' flanking sequence of the *D15/Omp85* gene. This sequence has been proven to be efficacious for up-regulation of the expression of *D15/Omp85* in *Neisseria meningitidis* and will be tested for the up-regulation of the expression of other *Neisseria* antigens. Primers used for the

amplification were **BAD 20** and **CM-PORA-D153** (5'- GGG CTC GAG TGT CAG TTC CTT GTG GTG C-3') containing *XhoI* restriction sites (underlined). This PCR fragment was cloned in the circle PCR plasmid obtained with primers CIRC1-PQ-Bgl and CIRC2-PQ-X. This plasmid can be used to transform *Neisseria meningitidis* serogroup B (*cps*-) and (*cps*-, *porA*-) strains. Integration by double crossing-over in the upstream region of *pilQ* will direct the insertion of the *porA* promoter 400bp upstream the *pilQ* ATG.

**Example 17:** Construction of a *kanR/sacB* cassette for introducing "clean", unmarked mutations in the *N. meningitidis* chromosome.

The aim of the experiment is to construct a versatile DNA cassette containing a selectable marker for the positive screening of recombination in the chromosome of *Neisseria meningitidis* (ie: *kanR* gene), and a counter selectable marker to delete the cassette from the chromosome after recombination (ie: *sacB* gene). By this method, any heterologous DNA introduced during homologous recombination will be removed from the *Neisseria* chromosome.

A DNA fragment containing the *neoR* gene and the *sacB* gene expressed under the control of its own promoter was obtained by restriction of the pIB 279 plasmid (Blomfield IC, Vaughn V, Rest RF, Eisenstein BI (1991), Mol Microbiol 5:1447-57) with *BamHI* restriction enzyme. The recipient vector was derived from plasmid pCMK, previously described. The *kanR* gene of the pCMK was deleted by restriction with enzymes *NruI* and *EcoRV*. This plasmid was named pCMKs. The *neoR/sacB* cassette was inserted in the pCMKs at a *BglII* restriction site compatible with *BamHI* ends.

*E. coli* harboring the plasmid is unable to grow in the presence of 2% sucrose in the culture medium, confirming the functionality of the *sacB* promoter.

This plasmid contains recombinogenic sequences allowing the insertion of the cassette at the *porA* locus in the chromosome of *Neisseria meningitidis* serogroup B. Recombinant *Neisseria* were obtained on GC agar plates containing 200µg/ml of kanamycin. Unfortunately, the *sacB* promoter was not functional in *Neisseria meningitidis*: no growth difference was observed on GC agar plates containing 2% sucrose.

A new cassette was constructed containing the *sacB* gene under the control of the *kanR* promoter. A circle PCR was performed using the plasmid pUC4K ((Amersham Pharmacia Biotech, USA)) as a template with **CIRC-Kan-Nco** (5'-CAT GCC ATG GTT AGA AAA ACT CAT CGA GCA TC-3') & **CIRC-Kan-Xba** (5'-CTA GTC TAG ATC AGA ATT GGT TAA TTG GTT G-3') oligonucleotides containing *NcoI* and *XbaI* restriction sites (underlined). The resulting PCR fragment was gel-purified, digested with *NcoI* and ligated to the *sacB* gene generated by PCR from the pIB279 plasmid with **SAC/NCO/NEW5**

(5'-CAT GCC ATG GGA GGA TGA ACG ATG AAC ATC AAA AAG TTT GCA A-3') oligonucleotide containing a *NcoI* restriction site (underlined) and a RBS (bold) & **SAC/NCO/NEW3** (5'-GAT CCC ATG GTT ATT TGT TAA CTG TTA ATT GTC-3') oligonucleotide containing a *NcoI* restriction site (underlined). The recombinant *E. coli* clones can be tested for their sensitivity on agar plates containing 2% sucrose. The new *kanR/sacB* cassette can be subcloned in the pCMKs and used to transform a *Neisseria meningitidis* serogroup B *cps*- strain. The acquired sucrose sensitivity will be confirmed in *Neisseria*. The pCMKs plasmid will be used to transform the recombinant *kanR/SacB Neisseria* to delete the entire cassette inserted in the chromosome at the *porA* locus. Clean recombinant *Neisseria* will be obtained on GC agar plates containing 2% sucrose.

**Example 18: Use of small recombinogenic sequences (43bp) to allow homologous recombination in the chromosome of *Neisseria meningitidis*.**

The aim of the experiment is to use small recombinogenic sequences (43bp) to drive insertions, modifications or deletions in the chromosome of *Neisseria*. The achievement of this experiment will greatly facilitate future work, in terms of avoiding subcloning steps of homologous sequences in *E. coli* (recombinogenic sequences of 43bp can easily be added in the PCR amplification primer). The *kanR* gene was PCR amplified from plasmid pUC4K with oligonucleotides **Kan-PorA-5** (5'-GCC GTC TGA ACC CGT CAT TCC CGC GCA GGC GGG AAT CCA **GTC CGT TCA GTT TCG GGA AAG CCA CGT TGT GTC**-3') containing 43bp homologous to the 5' flanking sequence of *NmB porA* gene (bold) and an uptake sequence (underlined) & **Kan-PorA-3** (5'-TTC AGA CGG CGC AGC AGG AAT



TTA TCG GAA ATA ACT GAA ACC GAA CAG ACT AGG CTG AGG TCT  
GCC TCG-3') containing 43bp homologous to the 3' flanking sequence of *NmB porA*  
gene (bold) and an uptake sequence (underlined). The 1300bp DNA fragment  
obtained was cloned in pGemT vector (Promega, USA). This plasmid can be used to  
transform a *Neisseria meningitidis* serogroupB *cps*- strain. Recombinant *Neisseria*  
will be obtained on GC plates containing 200µg/ml kanamycin. Integrations resulting  
from a double crossing-over at the *porA* locus will be screened by PCR with primers  
PPA1 & PPA2 as described previously.

**Example 19: Active protection of mice immunized with WT and recombinant *Neisseria meningitidis* blebs**

Animals were immunised three times (IP route) with 5 µg of the different  
OMVs adsorbed on Al(OH)<sub>3</sub> on days 0, 14 and 28. Bleedings were done on days 28  
(day 14 Post II) and 35 (day 7 post III), and they were challenged on day 35 (IP  
route). The challenge dose was 20 x LD<sub>50</sub> (~10<sup>7</sup> CFU/mouse). Mortality rate was  
monitored for 7 days after challenge.

OMVs injected were:

Group1: Cps-, PorA+ blebs

Group2: Cps-, PorA- blebs

Group3: Cps-, PorA-, NspA+ blebs

Group4: Cps-, PorA-, Omp85+ blebs

Group5: Cps-, PorA-, Hsf+ blebs

Figure 15 illustrates the pattern of these OMVs by analyzed SDS Page  
(Coomassie staining).

24 hours after the challenge, there was 100% mortality (8/8) in the negative  
control group (immunised with Al(OH)<sub>3</sub> alone) while mice immunised with the 5  
different OMVs preparations were still alive (7 to 8/8 mice survived). Sickness was  
also monitored during the 7 days and the mice immunised with the NSPA over-  
expressed blebs appeared to be less sick than the other groups. PorA present in PorA+  
blebs is likely to confer extensive protection against infection by the homologous  
strain. However, protection induced by PorA- up-regulated blebs is likely to be due at  
least to some extent, to the presence of increased amount of NspA, Omp85 or Hsf.



**Example 20: Immunogenicity of recombinant blebs measured by whole cell & specific ELISA methods**

To measure the ability of the antibodies to recognize the antigens present on the MenB cell surface, the pooled mice sera (from Example 19) were tested by whole cell ELISA (using tetracyclin inactivated cells), and titers were expressed as mid-point titers. All types of bleb antibodies induce a high whole cell Ab titer while the negative control group was clearly negative.

Blebs	WCE(H44/76) mid-point titer	
	14P2	14P3
CPS(-) PorA(+)	23849	65539
CPS(-) PorA(-)	20130	40150
CPS(-) PorA(-) NSPA(+)	8435	23846
CPS(-) PorA(-) OMP85(+)	4747	16116
CPS(-) PorA(-) HSF(+)	6964	22504
(-)	51	82

The specific Ab response to available recombinant HSF protein was carried out. Microplates were coated with 1 µg/ml full length HSF molecule.

The results illustrated in Figure 16 show that there was a good specific HSF response when HSF over-expressed OMVs were used to immunize mice (using purified recombinant HSF on the plates). The HSF over-expressed blebs induce a good level of specific antibodies.

**Example 21: The immunostimulant effect of *Moraxella catarrhalis* outer membrane vesicles (OMV or Blebs) evaluated on *Haemophilus influenzae* protein D (PD), alone or conjugated to *Streptococcus pneumoniae* polysaccharides (Spn 11V-PD)**

The immunostimulant effect of *Moraxella catarrhalis* outer membrane vesicles (OMV or Blebs) was evaluated on *Haemophilus influenzae* protein D (PD), alone or conjugated to *Streptococcus pneumoniae* polysaccharides (Spn 11V-PD).

## 5 Experimental procedure

Groups of 18 mice were subcutaneously immunized on day 0 and 14. Protein D (10µg) and the Spn 11V-PD conjugate (1 human dose) were injected either alone or adjuvanted with *Moraxella* blebs (10µg). On day 20, 27 or 35, mice were bled and anti-protein D titres were measured in an ELISA using purified recombinant protein D. The titres are defined as mid-point titres calculated by 4-parameter logistic model using the XL Fit software.

## Results

Serum antibody titers against PD

Antigens	Geometric mean titre (CI 95%)
PD <sup>a</sup>	228 (138-376)
PD + <i>M. catarrhalis</i> Blebs <sup>a</sup>	2871 (1476-5586)
<i>M. catarrhalis</i> Blebs <sup>a</sup>	52 (19-139)
Spn 11V-PD <sup>a</sup>	2161 (989-4719)
Spn 11V-PD + <i>M. catarrhalis</i> Blebs <sup>a</sup>	11518 (6960-19060)
<i>M. catarrhalis</i> Blebs <sup>a</sup>	71 (22-230)
Spn 11V-PD <sup>b</sup>	39498 (28534-54676)
Spn 11V-PD + <i>M. catarrhalis</i> Blebs <sup>b</sup>	55110 (45188-67210)
<i>M. catarrhalis</i> Blebs <sup>b</sup>	66 (53-81)
Spn 11V-PD <sup>c</sup>	94570 (65387-136778)
Spn 11V-PD + <i>M. catarrhalis</i> Blebs <sup>c</sup>	63310 (48597-82478)
<i>M. catarrhalis</i> Blebs <sup>c</sup>	58 (42-80)

a, animals were bled on day 21

b, animals were bled on day 27

c, animals were bled on day 35

It can be observed that when antigens are formulated with a bleb adjuvant in a vaccine, this vaccine can induce a faster immune response against the antigen (as well as a larger response). The adjuvant is therefore particularly suitable for vaccines for the elderly (over 55 years of age). The PD immunogenicity (and protective capacity against *Haemophilus influenzae*) may be significantly enhanced by the presence of blebs as an adjuvant.

## SEQ. ID NO:1

## Nucleotide sequence of the pCMK(+) vector

TCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCTGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGT  
AATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAGGCCAGCAAAAAGGCCAGGAACCGTA  
5 AAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAATCGACGCTCAAGTCAGAGG  
TGGCGAAACCCGACAGGACTATAAAGATAACAGGCGTTTTCCCCCTGGAAGCTCCCTCGTGCCTCTCCTGTTCCGACCCCT  
GCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCA  
GTTCCGGTGTAGGTCTGCTCCAAGCTGGGCTGTGTGCACGAACCCCCGTTTACGCCCCGACCGCTGCGCCTTATCCGGT  
AATATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGC  
10 GAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCT  
GCGCTCTGCTGAAGCCAGTTACCTTCGGAAGAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGT  
GGTTTTTTTGTGCAAGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCT  
TGACGCTCAGTGGAACGAAACTCACGTTAAGGGATTTTGGTTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTT  
TAAATTAAAAATGAAGTTTTTAAATCAATCTAAAGTATATATGAGTAACTTGGTCTGACAGTTACCAATGCTTAATCAGT  
15 GAGGCACCTATCTCAGCGATCTGTCTATTTCTGTTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACG  
GGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAA  
ACCAGCCAGCCGGAAGGGCCGAGCGCAGAAAGTGGTCTTGCAACTTTATCCGCCCTCCATCCAGTCTATTAATTGTTGCCGG  
GAAGCTAGAGTAAGTAGTTCCGCAGTTAATAGTTTGCACAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTACGCTC  
GTCGTTTGGTATGGCTTCATTACGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAG  
20 CGGTTAGCTCCTTCGGTCTCCGATCGTTGTCAGAAGTAAGTTGGCCGAGTGTTTACTCATGGTTATGGCAGCACTG  
CATAATTCTCTTACTGTATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATA  
GTGTATGCGGCGACCGAGTTGCTCTTGCCCGCGCTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAAGTGC  
TCATCATTGGAAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACT  
CGTGACCCCAACTGATCTTCAGCATCTTTTACTTTTACCAGCGTTTCTGGGTGAGCAAAAAACAGGAAGGCAAAATGCCGC  
25 AAAAAAGGGAATAAGGGCGACACGGAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTATCAGG  
GTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGA  
AAAGTGCCACCTGACGTCTAAGAAACCATTAATTATCATGACATTAACTATAAAAAATAGGCGTATCACGAGGCCCTTTTCG  
TCTCGCGCTTTTCGGTGATGACGGTGAAAACTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGG  
ATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTGGCGGGTGTGCGGGCTGGCTTAACTATGCGGCATCA  
30 GAGCAGATTGTACTGAGAGTGCACCATAAAATTGTAAACGTTAATATTTTGTAAAATTTCGCGTTAAATTTTTGTAAAT  
CAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGCCCCGAGATAGGGTTGAGTG  
TTGTTCCAGTTTGAACAAGAGTCCACTATTAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGC  
GATGGCCCACTACGTGAACCATCACCCAAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCCTAA  
AGGGAGCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGAAAGCGAAAGGAGCGG  
35 GCGCTAGGGCGCTGGCAAGTGTAGCGGTACGCTGCGCGTAACCACCACACCCGCCGCTTAATGCGCCGCTACAGGGC  
GCGTACTATGGTTGCTTTGACGTATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAAATACCGCATCAGGCGCCAT  
TCGCCATTACAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGG  
ATGTGCTGCAAGGCGATTAAAGTTGGGTAAAGCCAGGGTTTTCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAAGC  
TTGCCGTCTGAATACATCCCGTCATTCTCTCAAAAAACAGAAAACCAAAATCAGAAACCTAAAAATCCCGTCATTCCCGCGCA  
40 GGCGGGAATCCAGTCCGTTTCAAGTTTCGGTCATTTCCGATAAAATTCCTGCTGCTTTTCAATTTCTAGATTCCCACTTTCGTG  
GGAATGACGGCGGAAGGGTTTTGGTTTTTTCCGATAAAATTCCTGAGGCAATTGAAATTTCTAGATTCCCGCCTGCGCGGGAA  
TGACGGCTGTAGATGCCCGATGGTCTTTATAGCGGATTAACAAAAATCAGGACAAGGCGACGAAGCCGACAGTACAG  
ATAGTACGGAACCGATTCACTTGGTGCTTCAGCACCTTAGAGAATCGTTCTCTTTGAGCTAAGGCGAGGCAACGCCGTAC  
TTGTTTTTGTAAATCCACTATAAAGTGCCGCGTGTGTTTTTTATGGCGTTTTTAAAAAGCCGAGACTGCATCCGGGCAGC  
45 AGCGCATCGGCCCGCACGAGGTCTCTGGAGTCGCGAGCATCAAGGGCGAATTCTGCAGGGGGGGGGGGGAAAGCCACGTT  
GTGTCTCAAAATCTCTGATGTTACATTGCACAAGATAAAAAATATCATCATGAACAATAAACTGTCTGCTTACATAAA  
CAGTAATACAAGGGGTGTTATGAGCCATATTCAACGGGAACGCTCTGCTCGAGGCCGCGATTAAATTCCAACATGGATG

CTGATTTATATGGGTATAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCTATCGATTGTATGGGAAGCCC  
 GATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGATGAGATGGTCAGACTAACTG  
 GCTGACGGAATTTATGCCTCTTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTACTCACCCTGCGA  
 TCCCCGGGAAAACAGCATTCCAGGTATTAGAAGAATATCCTGATTGAGGTGAAAATATTGTTGATGCGCTGGCAGTGTT  
 5 CTGCGCCGGTTGCATTCGATTCTGTTTGTAAATTGTCCTTTTAAACAGCGATCGCGTATTTCTGCTCGCTCAGGCGCAATC  
 ACGAATGAATAACGGTTTGGTTGATGCGAGTGATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAG  
 AAATGCATAAGCTTTTGCCATTCTCACCAGATTGAGTCGTCACCTCATGGTGATTTCTCACTTGATAACCTTATTTTGGAC  
 GAGGGGAAATTAATAGGTTGTATTGATGTTGGACGAGTCGGAATCGCAGACCGGATACCAGGATCTTGCCATCCTATGGAA  
 CTGCCTCGGTGAGTTTTCTCCTTCATTACAGAAACGGCTTTTTTCAAAAATATGGTATTGATAATCCTGATATGAATAAAT  
 10 TGCAGTTTCATTTGATGCTCGATGAGTTTTTCTAATCAGAATTGGTTAATTGGTTGTAACACTGGCAGAGCATTACGCTG  
 ACTTGACGGGACGGCGGCTTTGTTGAATAAATCGAACTTTTGCTGAGTTGAAGGATCAGATCACGCATCTTCCCGACAAC  
 GCAGACCGTTCCGTGGCAAAGCAAAAGTTCAAAATCACCACCTGGTCCACCTACAACAAAGCTCTCATCAACCGTGGCTC  
 CCTCACTTTCTGGCTGGATGATGGGGCGATTGAGGCTGGTATGAGTCAGCAACACCTTCTTCACGAGGCAGACCTCAGC  
 GCCCCCCCCCTGCAGGAGGTCTGCGCTTGAATTGTGTTGTAGAAACACAACGTTTTTGAaaaaataagctattgTTT  
 15 TATATCAAAATATAATCATTTTTTAAATAAAGGTTGCGGCATTTATCAGATATTTGTTCTGAAAAATGGTTTTTTGCGGG  
 GGGGGGGGTATAATTGAAGACGTATCGGGTGTGTTGCCCGGAATTGTGAGCGGATAACAATTCGATGTTTTTAGGTTTTTA  
 TCAAAATTTACAAAAGGAAGCCCATATGCATCCTAGGCCTATTAATATTCGGGAGTATACGTAGCCGGCTAACGTTAACAA  
 CCGGTACCTCTAGAACTATAGCTAGCATGCGCAAAATTTAAAGCGCTGATATCGATCGCGCGCAGATCTGATTAAATAGGC  
 GAAATACCAGCTACGATCAAAATCATCGCCGGCGTTGATTATGATTTTTTCCAAACGCACTTCCGCCATCGTGTCTGGCGC  
 20 TTGGCTGAAACGCAATACCGGCATCGGCAACTACACTCAAATTAATGCCGCTCCGTCGGTTTGCGCCACAAATCTAAA  
 TATCGGGGCGGTGAAGCGGATAGCTTTGTTTTTGACGGCTTCGCCTTCATTCTTTGATTGCAATCTGACTGCCAATCTGC  
 TTCAGCCCCAAACAAAAACCCGGATACGGAAGAAAAACGGCAATAAAGACAGCAAAATACCGTCTGAAAGATTTTCAGACG  
 GTATTTTCGCATTTTTGCGTTGGTTTGCACATATAGTGAGACCTTGGCAAAAATAGTCTGTAAACGAAATTTGACGCATAA  
 AAATGCGCCAAAAAATTTCAATTGCCTAAACCTTCCTAATATTGAGCAAAAAGTAGGAAAAATCAGAAAAGTTTTGCA  
 25 TTTTGAaaATGAGATTGAGCATAAAATTTTAGTAACCTATGTTATTGCAAAAGGTCTCGAATTGTCATTCCACGCAGGCG  
 GGAATCTAGTCTGTTTCGGTTTCAGTTATTTCCGATAAAATCCTGCTGCGCCGTCTGAAGAATTCGTAATCATGGTCATAG  
 CTGTTTCTGTGTGAAATTGTTATCCGCTCACAATTCACACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGG  
 TGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCCGCTTTCCAGTCGGGAAACCTGTGCTGCCAGC  
 TGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTTGCGTATTGGGCGC  
 30

SEQ. ID NO:2

**Nucleotide sequence of DNA region (997 bp) up-stream from the NspA gene in the *Neisseria meningitidis* serogroup A strain Z2491.**

GGAACCGAACACGCCGTTTCGGTCATACGCCGCCGAAAGGTTTGCCGCAAGACGAAGCCGCCCTCGACATCGAAGACGCGG  
 35 TACACGGCGCGCTGGAAAGCGCGGGTTTTGTCCACTACGAAACATCGGCTTTTGCGAAACCAGCCATGCAGTGCCGCCAC  
 AATTTGAACTACTGGCAGTTTCGGCGATTATTTAGGCATAGGCGCGGGCGCGCACGGCAAAATTTCTATCCCGACCGCAT  
 CGAGCGCACCGTCCGCCGCCGCCACCCCAACGACTACCTCGCCTTAATGCAAAACCGACCGAGCGAAGCCGTCGAACGCA  
 AAACCGTCGCCGCCGAAGATTTGCCGTTTGAATTCATGATGAACGCCCTGCGCCTGACCGACGGCGTACCCACCGCGATG  
 TTGCAGGAGCGCACGGGCGTACCGAGTGCCAAAAATCATGGCGCAATCGAAACGGCAAGGCAAAAAGGCCTGCTGGAAAC  
 40 CGACCCCGCCGTATTCCGCCCGACCGAAAAAGGACGCTTGTTTTTTAAACGATTTGCTGCAGTGTTTTTTATAGTGGAATTA  
 ACAAAAACAGTACGGCGTTGCCTCGCCTTAGCTCAAAGAGAACGATTCTCTAAGGTGCTGAAGCACCAAGTGAATCGGT  
 TCCGTACTATCTGTACTGTCTGCGGCTTCGTCGCCCTTGTCCTGATTTTTGTTAATCCACTATATAAGCGCAAAACAAATCG  
 GCGGCCGCCGGGAAAACCCCCCGAACGCGTCCGGAATATGCTTATCGATGGAAACGCGAGCCGCATCCCCGCCGG  
 GCGTTTCAGACGGCACAGCCGCCCGGAAATGTCCGACGCTTAAGGCACAGACGCACAAAAAACCGTATGCCTGCAC  
 45 CTGCAACAAATCCGACAGATACCGCTGTTTTTTTCCAAACGTTTGCAAGTTTACCCATCCGCCGCGTGATGCCGCCACCA



CCATTTAAAGGCAACGCGCGGGTTAACGGCTTTGCCG

SEQ. ID NO:3

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the D15/Omp85 gene in the *Neisseria meningitidis* serogroup B strain ATCC13090.**

5 ACCATTGCCGCCCCGCGCCGGCTTCCAAAGCGGCGACAAAATACAATCCGTCAACGGCACACCCGTTGCAGATTGGGGCAG  
CGCGCAAACCGAAATCGTCCTCAACCTCGAAGCCGGCAAAGTCGCCGTGGGTTTCAGACGGCATCAGGCGCGCAAACCGT  
CCGCACCATCGATGCCGCAGGCACGCCGGAAGCCGGTAAAATCGCAAAAAACCAAGGCTACATCGGACTGATGCCCTTTA  
AAATCACAAACCGTTGCCGGTGGCGTGGAAAAAGGCAGCCCCGCCGAAAAAGCAGGCCTGAAACCGGGCGACAGGCTGACT  
10 GCCGCCGACGGCAAACCCATTACCTCATGGCAAGAATGGGCAAACCTGACCCGCCAAAGCCCCGGCAAAAAAATCACCCCT  
GAACTACGAACGCGCCGGACAAACCCATACCGCCGACATCCGCCCCGATACTGTGCAACAGCCCGACCACACCCTGATCG  
GGCGCGTCGGCCTCCGTCCGCAGCCGGACAGGGCGTGGGACGCGCAAATCCGCCGAGCTACCGTCCGTCTGTTATCCGC  
GCATTGCGCATGGGCTGGGAAAAAACCGTTTCCCACTCGTGGACAACCCTCAAATTTTTTCGGCAAACTAATCAGCGGCAA  
CGCCTCCGTGAGCCATATTTCCGGGCCGCTGACCATTGCCGACATTGCCGGACAGTCCGCCGAACTCGGCTTGCAAAGTT  
15 ATTTGGAATTTTTTGGCACTGGTCAGCATCAGCCTCGGCGTGTGTAACCTGCTGCCCGTCCCCGTTTGGACGGCGGCCAC  
CTCGTGTTTTATACTGCCGAATGGATACGCGGCAAACCTTTGGGCGAACGCGTCCAAAACATCGGTTTGCGCTTCGGGCT  
TGCCCTCATGATGCTGATGATGGCGGTGCGCTTCTTCAACGACGTTACCCGGCTGCTCGGTTAGATTTTACGTTTCGGAA  
TGCCGTCTGAAACCGCATTCGCGACCAACAAGGAAGTACA

20 SEQ. ID NO:4

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the Hsf-like gene from *Neisseria meningitidis***

ATTCCCGCGCAGGCGGGAATCCAGAAACGCAACGCAACAGGAATTTATCGGAAAAACAGAAACCTCACCGCCGTCATTC  
CCGCAAAGCGGGAATCTAGAAACACAACGCGGCAGGACTTTATCAGAAAAAACAGAAACCCACCGCCGTCATTCCCGC  
25 AAAAGCGGGAATCCAGACCCGTCGGCAGCGAAACTTACCGGATAAAACAGTTTCCTTAGATTCCACGTCCTAGATTCCCG  
CTTTCGCGGGAATGACGAGATTTTAGATTATGGGAATTTATCAGGAATGATTGAATCCATAGAAAAACCACAGGAATCTA  
TCAGAAAAACAGAAACCCCCACCGCGTCATTCCCGCGCAGGCGGGAATCCAGAAACACAACGCGGCAGGACTTTATCGG  
AAAAAACCGAAACCCACCGACCGTCATTCCCGCAAAAGTTGGAATCCAAAAACGCAACGCAACAGGAATTTATCGGAAA  
AAACAGAAACCCCCACCGCGTCATTCCCGCGCAGGCGGGAATCCAGAAACACAACGCAACAGGAATTTATCGGAAAAAAC  
30 AGAAACCCACCGACCGTCATTCCCGCAAAAGCGGGAATCCAGCAACCGAAAAACCACAGGAATCTATCAGCAAAAAACAG  
AAACCCCCACCGACCGTCATTCCCGCGCAGGCGGGAATCCAGAAACACAACGCGGCAGGACTTTATCGGAAAAAACAGAA  
ACCCACCGACCGTCATTCCCGCAAAAGCTGGAATCCAAAAACGCAACGCAACAGGAATTTATCGGAAAAAACAGAAACC  
CCACCGCCGTCATTCCCGCAAAAGCGGGAATCCAGACCCGTCGGCAGCGAAACTTACCGGATAAAACAGTTTCCTTAGAT  
TCCACGTCCAGATTCCCGCCTTCGCGGGAATGACGAGATTTTAAGTTGGGGGAATTTATCAGAAAAACCCCAACCCCA  
35 AAAACCGGGCGGATGCCGCACCATCCGCCCCCAACCCCGATTAAACCATCAAACAAACCAAAAGAAAAACAAA

SEQ. ID NO:5

**Nucleotide sequence of DNA region (772 bp ) up-stream from the PilQ gene from *Neisseria meningitidis***

40 GCGATGTCGGGAAGCCTTCTCCCGAATCATTACCCCTTGAGTCGCTGAAAATCGCCCAATCTCCGGAAAACGGCGGCAAT  
CATGACGGCAAGAGCAGCATCTGAACCTCAGTGCCATTGCCACCACCTACCAAGCAAATCCGTAGAAGAGCTTGCCGC  
AGAAGCGGCACAAAATGCCGAGCAAAAATAACTTACGTTAGGGAAACCATGAAACACTATGCCTTACTCATCAGCTTCT  
GGCTCTCTCCGCGTGTTCCTAAGGTTCTGAGGACCTAAACGAATGGATGGCACAAACGCGACGCGAAGCCAAAGCAGAAA  
TCATACCTTTCCAAGCACCTACCTGCGGTTGCGCCGGTATACAGCCCGCCGAGCTTACAGGGCCGAACGCATTCGAC  
45 TTCCGCCGCATGGAAACCGACAAAAAAGGGGAAAAATGCCCCCGACACCAAGCGTATTAAAGAAACGCTGGAAAAATTCAG  
TTTGAAAAATATGCGTTATGTGCGCATTTTGAAGTCTGGACAGAAAGTCTCCGGCTTCATCGAGGCTGAAGGTTATGTCT  
ACACTGTGCGGTGTCGGCAACTATTTGGGACAAAACTACGGTAGAATCGAAAGCATTACCGACGACAGCATCGTCCTGAAC  
GAGCTGATAGAAGACAGCACGGGCAACTGGGTTTCCCGTAAAGCAGAACTGCTGTTGAATTCTCCGACAAAAACACCGA  
ACAAGCGGCAGCACCTGCCGCAGAACAAAATTAAGAAGAGGATTACTCCATT

50

SEQ. ID NO:6



**Nucleotide sequence of DNA region (1000 bp ) up-stream from the Hap gene from *Neisseria meningitidis***

GTGCGGCAAAAAACAGCAAAAGCCCGCTGTCGATTGCCTGACCGTCCGCGTCCGTAAAAATCAGCATAGGTTGCCACGCGC  
 GGCTTGGGCGTTTTCCACACAAAGCCTCTGCCATCGGCAGCAGGTTTTTCCCGATATGCGTATCACGCCCACGCCGCC  
 5 GCGCCCGGGTGCGGTAGCGACTGCCGCAATCGTTGGAACGTTATCCGACATAAAACCCCGAAAATTCAAAACAGCCGCG  
 ATTATAGCAAATGCCGTCTGAAGTCCGACGGTTTTGGCTTTCAGACGGCATAAAACCGCAAAAATGCTTGATAAATCCGTC  
 CGCCTGACCTAATATAACCATATGGA AAAACGAAACACATACGCCCTTCCTGCTCGGTATAGGCTCGCTGCTGGGTCTGTT  
 CCATCCCGCAAAAACCGCCATCCGCCCCAATCCCGCCGACGATCTCAAAAACATCGGCGGCGATTTTCAACGCGCCATAG  
 AGAAAGCGCGAAAATGACCGAAAACGCACAGGACAAGGCGCGGCAGGCTGTGAAACCGTCGTCAAATCCCGGAGCTTG  
 10 TCGAGCAAATCCTGTCCGACGAGTACGTGCAAATAATGATAGCCCGGCGTTTTCCATTCGGGATCGTTGCCGCCGCCGTCC  
 GACTTGGCGCAATACAACGACATTATCAGCAACGGGGCAGACCGCATTATGGCAATGGCGGAAAAAGACAAGCCGTCCG  
 GCACGAAACCATAACGGCAAGACCAAACCTTCAACAGGCGCGGGCAACTGTACGGCTTCATCAGCGTCATCCTGATACTGC  
 TTTTGTCCGTCTTCTCGTATGGAGCGGCTACCCCGCAACCGCCGCTCCCTTGCCGGCGGCACAGTGGTTGCCTTGGCG  
 GGTGCTTTCGTGATTGGAAGAAGCCGAGACCAAGGCAAAAATTAATTGCAAATCCTAGGGCGTGCTTCATATCCGCCCGA  
 15 ACGCCGAACCGCACATATAGGCACATCCCGCGCGCCGCGGAAGCGGAAGCCGCGCCCTCCCAAACAAACCCGAATCCCG  
 TCAGATAAGGAAAAATA

SEQ. ID NO:7

**Nucleotide sequence of DNA region (924 bp) up-stream from the NspA gene from *Neisseria meningitidis* (serogroup B) (ATCC13090)**

GGAACCGAACACGCCGTTCCGGTCATACGCCGCCGAAAGGTTTGCCGCAAGACGAAGCCGCCCTCGACATCGAAGACGCGG  
 TACACGGCGCGCTGGAAGGCGCGGGTTTTGTCCACTACGAAACATCGGCTTTTGCGAAACCAGCCATGCAGTGCCGCCAC  
 AATTTGAACTACTGGCAGTTCGGCGATTATTTAGGCATAGGCGCGGGCGCTCACGGCAAAATTTCTATCCCGACCGCAT  
 CGAGCGCACCGTCCGCCCGCCGCAACCCCAACGACTACCTCGCCTTAATGCAAAGCCAACCGAGTGAAGCCGTCGAACGCA  
 25 AAACCGTTGCCGCCGAAGATTTGCCGTTTGAGTTCATGATGAACGCCCTGCGCCTGACCGACGCGTACCCGCCGCGATGT  
 TGCAGGAGCGCACGGGCGTACCGAGTGCCAAAATCATGGCGCAAAATCGAAACGGCAAGGCAAAAAGGCTGCTGGAAACC  
 GACCCCGCCGTATTCCGCCCGACCGAAAAAGGACGCTTGTTTTTAAACGATTTGCTGCAGTGTTTTTTATAGTGGATTAA  
 CAAAACACAGTACGGCGTTGCCCTCGCCTTAGCTCAAAGAGAAGCATTTCTTAAGGTGCTGAAGCACCAAGTGAATCGGTT  
 CCGTACTATTTGTACTGTCTGCGGCTTCGTGCGCTTGCTCGATTTTTGTAAATCCACTATATAAGCGCAAAACAAATCGG  
 30 CGGCCGCCCGGAAAAACCGCCCCGAACGCGTCCGGAATATGCTTATCGATGGAAAACGCAGCCGCATCCCCCGCCGG  
 GCGTTTCAGACGGCACAGCCGCCGCGGAAATGTCCGACGCTTAAGGCACAGACGCACACAAAACCGTATGCCTGCACCT  
 GCAACAATCCGACAGATACCGCTGTTTTTTCCAAACCGTTTGCA

SEQ. ID NO:8

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the FrpB gene from *Neisseria meningitidis* (serogroup B)**

AAGTGGGAATCTAAAAATGAAAAGCAACAGGAATTTATCGGAAATGACCGAAACTGAACGGACTGGATTCCCGCTTTTCGC  
 GGAATGACGGCGACAGGGTTGCTGTTATAGTGGATGAACAAAAACAGTACGTCGTTGCCTCGCCTTAGCTCAAAGAGA  
 ACGATTCTCTAAGGTGCTGAAGCACCAAGTGAATCGGTTCCGTCCTATTTGTACTGTCTGCGGCTTCGTCGCTTGTCTCT  
 40 GATTTCTGTTTCGTTTTTCGGTTATTCCCGATAAATTACCGCCGTTTCTCGTCATTTCTTAACCCCTTCGTCATTCCCGCGC  
 AGGCGGGAATCTAGTTTTTTTTGAGTTCAGTTGTTTCTGATAAATTTCTGACGCTTTGAGTTCCTAGATTCCCACTTTTCG  
 TGGGAATGACGGTGGAAAAGTTGCCGTGATTTCCGATAAATTTTCGTAACGCATAATTTCCGTTTTTACCCGATAAATGCC  
 CGCAATCTCAAATCCCGTCATTCCCCAAAAACAAAAATCAAAAACAGAAATATCGTCATTCCCGCGCAGGCGGGAATCT  
 AGACCTTAGAACAAACAGCAATATTCAAAGATTATCTGAAAGTCCGAGATTCTAGATTCCCACTTTTCGTGGGAATGACGAA  
 45 TTTTAGGTTTTCTGTTTTTGGTTTTCTGTCTTTCGCGGAATGATGAAATTTTAAGTTTTAGGAATTTATCGGAAAAACAG  
 AAACCGCTCCGCCGTCATTCCCGCACAGGCTTCGTCATTCCCGCGCAGGCTTCGTCATTCCCGCATTTGTTAATCCACTA  
 TATTCCCGCCGTTTTTTTACATTTCCGACAAAACCTGTCAACAAAAACAACACTTCGCAAATAAAAACGATAATCAGCTT  
 TGCAAAAATCCCCCCCCCTGTTAATATAAATAAAAATAATTAATTAATTTTCTTATCCTGCCAAATCTTAACGGT  
 50 TTGGATTTACTTCCCTTCATACACTCAAGAGGACGATTGA

SEQ. ID NO:9

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the FrpA gene from *Neisseria meningitidis* (serogroup B)**

CTATAAAGATGTAAATAAAAATCTCGGTAACGGTAACACTTTGGCTCAGCAAGGCAGCTACACCAAAACAGACGGTACAA  
 CCGCAAAAATGGGGGATTTACTTTTAGCAGCCGACAATCTGCACAGCCGCTTCACGAACAAAATGCTATCCATTAGCCAT  
 GTTCGGGAAAACACGATTTCCCGGTTTGTTTTAGGCTGTCTAAACAAATAACCATAAATGTATATCATTTTAAAAATAA  
 ATAAAAGTATTTAACTATTATTGACGAAATTTTAGAGAAAGAGTAGACTGTGATTAAATGACAAACAATAGTGAGAAAG  
 GAAATATTTACTATCCGAGCACAGAGCATATTTTAGGTAGCCTGTAAGTTCCTGCTGGCGGAAGAGGATGAAGGTGGA  
 60 CTTACCCGAGAATAAATGTCCTGTTGTGTGATATGGATGCCATGCCGCAAGCAATTGATGCAATCACGGCAGTCCTACT  
 TGAATGAAACCTGTGCTTGCAGAAATTTGAAAACGCTATTTTTAAGAAAGGATAAAGGGAGAAAGAATTTTGGTTTTTAA  
 GCTGCATGAAACCGTGTGGAATAAATGCACACCTACGATAATTAATAATTTTCGTTTTTTATTCTACAAGCTATTTATA  
 TATGATTGCTAAAAGTTTATTTTTTAGATGCCAAAAATATATTTTATATACTTCATATTGTTTATATGTCCTTATTTGA  
 ATATATCTTACGATGGGGAAATATTTATATATTTTATAATAAATTTTACTCATTTGCTAATATGTCATGGAATATTACTT  
 65 GTATTTTGTAGAATTTTCCATATGAAAATATTCATTTACTATTTTCTGAAGTTTATTAGTTTATTTTAAATATTTT

ACCTCTTATATTTACCATAAGAGAGCTAATTGATTCATATTATATTGAGTCGATAATTAATTTATTCTTAATTTTAATTC  
CTCACGTTATTTTTTTAATTTACTTGAAAGGAAAGCAGAT

SEQ. ID NO:10

5 **Nucleotide sequence of DNA region (1000 bp ) up-stream from the FrpC gene from *Neisseria meningitidis* (serogroup B)**

10 GGAAACAGAGAAAAAGTTTCTCTTCTATCTTGGATAAATATATTTACCCTCAGTTTAGTTAAGTATTGGAATTTATACC  
TAAGTAGTAAAAGTTAGTAAATTATTTTTAACTAAAGAGTTAGTATCTACCATAATATATTCTTTAACTAATTTCTAGGC  
TTGAAATTATGAGACCATATGCTACTACCATTTATCAACTTTTTATTTTGTATTGGGAGTGTTTTTACTATGACCTCA  
15 TGTGAACCTGTGAATGAAAAGACAGATCAAAAAGCAGTAAGTGCAGCAACAGGCTAAAGAACAAACCAGTTTCAACAATCC  
CGAGCCAATGACAGGATTTGAACATACGGTTACATTTGATTTTCAGGGCACCAAAATGGTTATCCCCCTATGGCTATCTTG  
CACGGTATACGCAAGACAATGCCACAAAATGGCTTCCGACACGCCCCGGGCAGGATGCTTACTCCATTAATTTGATAGAG  
ATTAGCGTCTATTACAAAAAACCGACCAAGGCTGGGTCTTGAGCCATACAACCAGCAAAACAAAGCACACTTTATCCA  
ATTTCTACGCGACGGTTTGGATAGCGTGGACGATATTGTTATCCGAAAAGATGCGTGTAGTTTAAGTACGACTATGGGAG  
20 AAAGATTGCTTACTTACGGGGTTAAAAAATGCCATCTGCCTATCCTGAATACGAGGCTTATGAAGATAAAAGACATATT  
CCTGAAAAATCCATATTTTCATGAATTTTACTATATTA AAAAAGGAGAAAATCCGGCGATTATTACTCATCGGAATAATCG  
AATAAACCAAACTGAAGAAGATAGTTATAGCACTAGCGTAGGTTCTGTATTAAACGGTTTCACGGTACAGTATTACCCGT  
TTATTCCGGGAAAAGCAGCAGCTCACACAGCAGGAGTTGGTAGGTTATCACCACAAGTAGAGCAATTGGTACAGAGTTT  
GTAAACAATTCAAATAAAAAATAATTTAAAGGATCTTATT

SEQ. ID NO:11

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the Omp85 gene from *Neisseria meningitidis* (serogroup B)**

25 ACGTCCGAACCGTGATTCCGCAACGCGCGCCCAAACCAAAGCCCAAGCCAAAATGCCGATATAGTTGGCATTGGCAAT  
CGCGTTAATCGGGTTGGCGACCAGGTTTCATCAGCAGCGATTTCAACACTTCCACAATGCCGGAAGGCGGCGCGGCGGACA  
CATCGCCCGCGCCCGCCAAAACAATGTGCGTCGGGAAAACCATAACCGGCGATGACGGCGGTGAGGGCTGCGGAAAACGTA  
CCAATGAGGTAAAGGATGATAATCGGCCTGATATGCGCCTTGTGCTTTTTGGTGCTGCGCGATTGTGGCCGCCACCAA  
AATAAATACCAAACCGGCGCGACCGCTTTGAGCGCGCCGACAAACAGGCTGCCGAACAAGCCTGCCGCAAGCCAGTT  
30 GCGGGGAAACCGAACCAGTTACGATGCCCCAACGCCAAACCGGCGGCAATCTGCCTGACCAGGCTGACGCGGCCGATCGCA  
TGAAATAAGGATTTGCCGAACGCCATAATCTTCCTTATGTTGTGATATGTTAAAAAATGTTGTATTTTAAAGAAAAC  
CATTCTCTGTGTTTTTTTTATTTTTCGGCTGTGTTTTAAGGTTGCGTTGATTTGCCCTATGCAGTGCCGGACAGGCTTTG  
CTTTATCATTTCCGCGCAACGGTTTAATTTATTGAACGAAAATAAATTTATTTAATCCTGCCTATTTTCCGGCACTATTCC  
GAAACGCGAGCCTGTTTTCCATATGCGGATTGGAAACAAAATACCTTAAACAAGCAGATACATTTCCGGCGGGCCGCAAC  
35 CTCCGAAATACCGGCGGCAGTATGCCGTCTGAAGTGTCCCGCCCCGTCCGAACAACACAAAAACAGCCGTTGAAACCCCT  
GTCCGAACAGTGTTAGAATCGAAATCTGCCACACCGATGCACGACACCCGTACCATGATGATCAAACCGACCGCCCTGCT  
CCTGCCGGCTTTATTTTTCTTTCCGCACGCATACGCGCCT

SEQ. ID NO:12

**Nucleotide sequence of DNA region (772 bp ) up-stream from the PilQ gene from *Neisseria meningitidis* (serogroup B) (ATCC13090)**

40 GCGATGTCGGGAAGCCTTCTCCGAATCATTACCCCTTGAGTCGCTGAAAATCGCCCAATCTCCGGAAAACGGCGGCAAT  
CATGACGGCAAGAGCAGCATCCTGAACCTCAGTGCCATTGCCACCACCTACCAAGCAAAATCCGTAGAAGAGCTTGCCGC  
AGAAGCGGCACAAAATGCCGAGCAAAAATAACTTACGTTAGGGAAACCATGAAACACTATGCCTTACTCATCAGCTTTCT  
GGCTCTCTCCGCGTGTTCCTCAAGGTTCTGAGGACCTAAACGAATGGATGGCACAAACGCGACGCGAAGCCAAAGCAGAAA  
45 TCATACCTTTTCCAAGCACCTACCCTGCCGGTTGCGCCGGTATACAGCCCGCCGAGCTTACAGGGCCGAACGCATTTCGAC  
TTCCGCGCATGGAAACCGACAAAAAAGGGGAAAATGCCCCCGACACCAAGCGTATTAAAGAAACGCTGGAAAAATTCAG  
TTTGAAAATATGCGTTATGTCGGCATTTTGAAGTCTGGACAGAAAGTCTCCGGCTTCATCGAGGCTGAAGGTTATGTCT  
ACACTGTCGGTGTTCGGCAACTATTTGGGACAAAACCTACGGTAGAATCGAAAGCATTACCGACGACAGCATCGTCCTGAAC  
GAGCTGATAGAAGACAGCACGGGCAACTGGGTTTCCCGTAAAGCAGAACTGCTGTTGAATTCTTCCGACAAAAACACCGA  
50 ACAAGCGGCAGCACCTGCCGCAACAAAATTAAGAAGAGGATTACTCCATT

SEQ. ID NO:13

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the Hsf-like gene from *Neisseria meningitidis* (serogroup B)**

55 TTTGTTTTTTCTTTTGGTTTGTGTTGAATGGTTAAATCGGGGTTTGGGGCGGATGGTGCGGCATCCGCCCCGTTTGGG  
GGTTGGGGGTTTCTGATAAATCCCCCACTTAAATCTCGTCATTCCCGCGAAGGCGGGAATCTGGGACGTGGAATCT  
AAGGAAACTGTTTTATCCGGTAAGTTTCCGTGCCGACGGGTCTGGATTCCCGCTTTTGGCGGAATGACGGCGGTGGGGTT  
TCTGTTTTTTCCGATAAATTCCTGTTGCGTTGCGTTTTTGGATTCCAGCTTTTGGCGGAATGACGGTCGGTGGGGTTTCT  
GTTTTTTCCGATAAAGTCTGCGCGTGTGTTTTCTGGATTCCCGCTGCGCGGGAATGACGGTCGGTGGGGTTTCTGTT  
60 TTTTGTGATAGATTCTGTGGTTTTTCGGTTGCTGGATTCCCGCTTTTGGCGGAATGACGGTCGGTGGGGTTTCTGTTT  
TTTCCGATAAATTCCTGTTGCGTTGTGTTTTCTGGATTCCCGCTGCGCGGGAATGACGGTCGGTGGGGTTTCTGTTTTTC  
CGATAAATTCCTGTTGCGTTGCGTTTTTGGATTCCAACTTTTGCGGGAATGACGGTCGGTGGGGTTTCTGTTTTTCCGA  
TAAAGTCTGCGCGTGTGTTTTCTGGATTCCCGCTGCGCGGGAATGACGGTCGGTGGGGTTTCTGTTTTTCTGATAGA  
TTCTGTGGTTTTTCTATGGATTCAATCATTCCTGATAAATCCCATAATCTAAATCTCGTCATTCCCGCGAAAGCGGG  
65 AATCTAGGACGTGGAATCTAAGGAACTGTTTTATCCGGTAAGTTTCCGTGCCGACGGGTCTGGATTCCCGCTTTTGGCG  
GAATGACGGCGGTGGGGTTTCTGTTTTTCTGATAAAGTCCGCGCTTGTGTTTCTAGATTCCCGCTTTTGGCGGAAT  
GACGGCGGTGAGGTTTCTGTTTTTTCCGATAAATTCCTGT

SEQ. ID NO:14

70 **Nucleotide sequence of DNA region (1000 bp ) up-stream from the Hap gene from *Neisseria meningitidis* (serogroup B)**



AATCAGCATAGGTTGCCACGCGCGGCTTGGGCGTTTTCCACACAAAGCCTCTGCCATCGGCAGCAGGTTTTTCCCGAT  
ATGCGTATCACGCCCACGCGCGCGCGCGGGTGCAGTACGACTGCCGCAATCGTTGGAACGTTATCCGACATAAAACC  
CCCGAAAATTCAAAACAGCCGCGATTATAGCAAATGCCGTCTGAAGTCCGACGGTTTGGCTTTCAGACGGCATAAAACCG  
5 CAAAAATGCTTGATAAATCCGTCGCGCTGACCTAATATAACCATATGGAAAAACGAAACACATACGCCTTCCTGCTCGGT  
ATAGGCTCGCTGCTGGGTCTGTTCCATCCCGCAAAACCGCCATCCGCCCCAATCCCGCCGACGATCTCAAAAACATCGG  
CGGCGATTTTCAACGCGCCATAGAGAAAGCGCGAAATGACCGAAAACGCACAGGACAAGGCGCGGAGGCTGTCGAAAC  
CGTCGTCAAATCCCCGGAGCTTGTGAGCAAATCCTGTCCGACGAGTACGTGCAAATAATGATAGCCCGCGTTTCCATT  
CGGGATCGTTGGCCGCGCGTCCGACTTGGCGCAATACAACGACATTATCAGCAACGGGGCAGACCGCATTATGGCAATG  
10 GCGGAAAAAGAACAGCCGTCCGGCACGAAACCATACGGCAAGACCAACCTTCAACAGGCGCGGGCAACTGTACGGCTT  
CATCAGCGTCATCCTGATACTGCTTTTTGCGCTCTTCTCGTATGGAGCGGCTACCCCGCAACCGCGCCTCCCTTGCCG  
GCGGCACAGTGGTTGCCTTGGCGGGTGTCTTCGTGATTGGAAGAAGCCGAGACCAAGGCAAAAATTAATTGCAAATCCTA  
GGGCGTGTTCATATCCGCCGAACGCCGAACCGCACATATAGGCACATCCCGCGCGCCGCGGAAGCGGAAGCCGCGCC  
CTCCCAAACAAACCCGAATCCCGTCAGATAAGGAAAAATA

15 SEQ. ID NO:15

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the LbpA gene  
from *Neisseria meningitidis* (serogroup B)**

GATTTTGGTCATCCCGACAAGCTTCTGTGCAAGGGCGTGAAATTCCTTTGGTTAGCCAAGAGAAAACCATCAAGCTTGC  
CGATGGCAGGGAAATGACCGTCCGTGCTTGTGCGACTTTTTGACCTATGTGAAACTCGGACGGATAAAAACCGAACGCC  
20 CGGCAAGTAAACCAAAGGCGGAAGATAAAAGGGAGGATGAAGAGAGTGCAGGCGTTGGTAACGTCGAAGAAGGCGAAGGC  
GAAGTTCCGAAGATGAAGGCGAAGAAGCCGAAGAAATCGTCGAAGAAGAACCCGAAGAAGAAGCTGAAGAGGAAGAAGC  
TGAACCCAAAGAAGTTGAAGAAACCGAAGAAAAATCGCCGACAGAAGAAGCGGCAGCGGTTCAAACGCCATCCTGCCTG  
CCTCGGAAGCCTCTAAAGGCAGGGACATCGACCTTTTCTGAAAGGTATCCGCACGGCGGAAGCCGACATTCCAAGAACC  
GGAAAGCACACTATACCGGCACTTGGGAAGCGGTATCGGCACACCCATTCAATGGGACAATCAGGCGGATAAAGAAGC  
25 GGCAAAAGCAGAATTTACCGTTAATTTGCGCGAGAAATCGATTTCGGAACGCTGACGGAGAAAAACGGTGTACAACCTG  
CTTTCTATATTGAAAACGGCAAGATTGAGGGCAACGGTTTCCACGCAACAGCACGCACTCGTGAGAACGGCATCAATCTT  
TCGGGAAATGGTTCGACCAACCCCAAGACCTTCCAAGCTAGTGATCTTCGTGTAGAAGGAGGATTTTACGGCCCGCAGCG  
GAGGAATTGGGCGGTATTATTTTCAATAAGGATGGGAAATCTCTTGGTATAACTGAAGGTACTGAAAATAAAGTTGAAGT  
TGAAGCTGAAGTTGAAGTTGAAGCTGAAACTGGTGTGTCGAACAGTTAGAACCTGATGAAGTTAAACCCCAATTTCGGCG  
30 TGGTATTCGGTGCGAAGAAAGATAATAAAGAGGTGAAAA

SEQ. ID NO:16

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the LbpB gene  
from *Neisseria meningitidis* (serogroup A)**

CGGCGTTAGAGTTTAGGGCAGTAAGGGCGCGTCCGCCCTTAGATCTGTAAGTTACGATTCGGTTAAATAACTTTTACTGA  
CTTTGAGTTTTTTGACCTAAGGGTGAAAGCACCTTACTGCTTAAAGTCCAACGACAAAAACCAAAGACAAAAACACTT  
TTATTACCCTAAAATCGAACACCCATAAATGACCTTTTTTGTCTTTGGCGAGGCGGCAGTAAGGGCGCGTCCGCCCTTAG  
ATCTGTAAGTTATGATTCCGTTAAATAGCCTTTACTGACTTTGAGTTTTTTGACCTAAGGGCGGACGCGCCCTTACTGCT  
TCACCTTCAATGGGCTTTGAATTTTGTTCGCTTTGGCTTGCTTGACCTAAGGGTGAAAGCACCTTACTGCTTCACTTCAATGGGCTT  
40 AAGACGAAAAGGGTTATTTACGGGGGTGGATTTTAGGCAGTAAGGGCGCGTCCGCCCTTAGATCTGTAAGTTATGATT  
CCGTTAAATAGCCTTTACTGACTTTGAGTTTTTTGACCTAAGGGTGAAAGCACCTTACTGCTTCACTTCAATGGGCTT  
TGAATTTTGTTCGCTTTGGCTTGCTTGATCTAAGGGTGAAAGCACCTTACTGCTTCACTTCAATGGGCTT  
TTTACGGCGTTAGAGTTTAGGGCAGTAAGGGCGCGTCCGCCCTTAGATCCAGACAGTCACGCCTTTGAATAGTCCATTTT  
GCCAAAGAACTCTAAAACGCAGGACCTAAGGGTGAAAGCACCTTACTGCTTACATCCAAGCACCTTACTGCACCACG  
45 TCCACGCACCTTACTGCCCTACGTCCACGCACCTTACTGCCCTACATCCAAGCACCTTACTGCCTTACATAGACATG  
ACAGACGCCGAGCAGCGGAACAGGACTAAAAACAATTAAGTGATATTTTTGCCCAACTATAATAGACATGTATAATTATA  
TTACTATTAATAATAATTAGTTTATCCTCCTTTTCATCCC

SEQ. ID NO:17

**Nucleotide sequence of DNA region (731 bp ) up-stream from the TbpA gene  
from *Neisseria meningitidis* (serogroup B) (ATCC13090)**

TATGAAGTCGAAGTCTGCTGTTCCACCTTCAATTATCTGAATTACGGAATGTTGACGCGC  
AAAAACAGCAAGTCCGCGATGCAGGCAGGAGAAAGCAGTAGTCAAGCTGATGCTAAACG  
GAACAAGTTGGACAAAGTATGTTCTCCAAAGGCGAGCGCACCGATGAAAAAGAGATTCCA  
55 AACGACCAAAACGTCGTTTATCGGGGGTCTTGGTACGGGCATATTGCCAACGGCACAAAGC  
TGGAGCGGCAATGCTTCCGATAAAGAGGGCGGCAACAGGGCGGACTTTACTGTGAATTTT  
GGTACGAAAAAATTAACGGCACGTTAACCGCTGACAACAGGCAGGCGGCAACCTTTACC  
ATTGTGGGCGATATTGAGGGCAACGGTTTTTCCGGTACGGCGAAAACTGCTGACTCAGGT  
TTTGATCTCGATCAAAGCAATAACACCCGCACGCCTAAGGCATATATCACAACGCCAAG  
60 GTGCAGGGCGGTTTTTACGGGCCCAAGCCGAAGAGTTGGGCGGATGGTTTGCCTATTTCG  
GACGATAAACAAACGAAAAATGCAACAGATGCATCCGGCAATGGAAATTCAGCAAGCAGT  
GCAACTGTGCTATTTCGGTGCGAACGCCAAAGCCTGTGCAATAAGCACGGTTGCCGAAC  
AATCAAGAATAAGGCCTCAGACGGCACCGCTCCTTCCGATACCGTCTGAAAGCGAAGAGT  
AGGGAAACACT

65 SEQ. ID NO:18

**Nucleotide sequence of DNA region (373 bp) up-stream from the OmpA gene  
from *Neisseria meningitidis* (serogroup B) (ATCC13090)**

CGTACCGCATTCGCGACTGCAGTGAAAAAGTATTGAAAGCAGTCGAAGCAGGCGATAAAGCTGCCGCACAAGCGGTTTA  
CCAAGAGTCCGTCAAAGTCATCGACCGCATCGCCGACAAGGGCGTGTTCATAAAAAACAAAGCGGCTCGCCACAAAACCC  
GTTTGTCTCAAAAAGTAAACCTTGGCTTGATTTTTGCAAAACCTGCAATCCGGTTTTTCATCGTCGATTCCGAAAACCCC  
TGAAGCCCGACGGTTTCGGGGTTTTCTGTATTGCGGGGACAAAATCCCGAAATGGCGGAAAGGGTGCGGTTTTTTATCCG  
AATCCGCTATAAATGCCGTCTGAAAACCAATATGCCGACAATGGGGGTGGAG

## SEQ. ID NO:19

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the Pla1 gene from *Neisseria meningitidis* (serogroup B)**

5 TTTTGGCTTCCAGCGTTTCATTGTTTTTCGTACAAGTCGTAAGTCAGCTTCAGATTGTTGG  
 CTTTTTTAAAGTCTTCGACCGTACTCTCATCAACATAGTTTCGACCGATTGTAGATGTTCA  
 GAGTATCGGTGGCAGCGGCTTCGGCATTGGCAGCAGACGCGAGCGTCTGCTTGAGGTTGCA  
 CGGCGTTTTTTTTTCGCTGCCGCCGAGGCTGCCAGAGACAGCGCGGCCAAAACGGCTAATA  
 CGGATTTTTTTCATACGGGCAGATTCTCTGATGAAAGAGGTTGGAAAAAAGAAATCCCCGC  
 10 GCCCCATCGTTACCCCGGCGCAAGGTTTGGGCATTGTAAAGTAAATTGTGCAAACTCAA  
 AGCGATATTGGACTGATTTTCCTAAAAAATTATCCTGTTTCCAAAAGGGGAGAAAAACGT  
 CCGCCCGATTGTCGCTTTTTTTCGCTGTGAGGTTGTCGACGGGCGGATAGAGAGAAA  
 AGGCTTGATATAATGTAAACCCCTTTAAATTGCGCGTTTACAGAAATTTATTTTTCTT  
 CCAGGAGATTCCAATATGGCAACAGCGCACAAAGCAGCAAAACGTGCCCGCCAGTCCGTC  
 15 AAACAACGCGCCCAATGCTAGCCTGCGTACCGCATTCCGCACCGCAGTGAAAAAAGTA  
 TTGAAAGCAGTCGAAGCAGGCGATAAAGCTGCCGACAAAGCGGTTTACCAAGAGTCCGTC  
 AAAGTCATCGACCGCATCGCCGACAAGGGCGTGTTCACAAAAAACAAGCGGCACGCCAC  
 AAAAGCCGTCTGTCTGCAAAAGTAAAAGCCTTGGGTTGATTTTGCAAAACCGCCAAGGC  
 GGTGATACGCGATAAGCGGAAAACCCCTGAAGCCCGACGGTTTCGGGGTTTTCTGTATTG  
 20 CGGGGGCAAAATCCCGAAATGGCGGAAAGGTTGCGATTTTTTATCCGAATCCGCTATAAA  
 ATGCCGTTTGAACCAATATGCCGACAATGGGGGCGGAG

## SEQ. ID NO:20

**Nucleotide sequence of DNA region (1000 bp) up-stream from the FhaB gene from *Neisseria meningitidis* (serogroup B)**

25 TACGGAACTGCAAGCGGATCCAGAAGTTACAGCGTGCATTATTCGGTGCCCGTAAAAAATGGCTGTTTTCTTTAATC  
 ACAATGGACATCGTTACCACGAAGCAACCGAAGGCTATTCCGTCAATTACGATTACAACGGCAAACAATATCAGAGCAGC  
 CTGGCCGCCGAGCGCATGCTTTGGCGTAACAGACTTCATAAACTTCAGTCGGAATGAAATTATGGACACGCCAAACCTA  
 TAAATACATCGACGATGCCGAAATCGAAGTGCACGCCGCCGCTCTGCAGGCTGGGAAGCCGAATTGCGCCACCGTGCTT  
 30 ACCTCAACCGTTGGCAGCTTGACGGCAAGTTGTCTTACAAACGCGGGACCGGCATGCGCCAAAGTATGCCTGCACCGGAA  
 GAAACCGCGCGGATATTCTTCCAGGTACATCTCGTATGAAATCATTACTGCCGTTTGGACGCGAGCCGCCCATTTAT  
 TTTAGGCAACAGCAGTTTTTCTACGCAACCGCATTCAGCTCAATGGAACAAAACGCCGTTGGTTGCCCAAGATAAAT  
 TGTCAATCGGCAGCCGCTACACCGTTCCGCGATTGATGGGGAGCAGAGTCTTTTCGGAGAGCGAGGTTTCTACTGGCAG  
 AATACTTTAACTTGGTATTTTCATCCGAACCATCAGTTCTATCTCGGTGCGGACTATGGCCGCGTATTTGGCGAAAGTGC  
 35 ACAATATGTATCGGGCAAGCAGCTGATGGGTGCAGTGGTCCGCTTCAGAGGAGGGCATAAAGTAGGCGGTATGTTGCTT  
 ATGATCTGTTTGGCGGCAAGCCGCTTCATAAACCCAAAGGCTTTCAGACGACCAACACCGTTTACGGCTTCAACTGAAT  
 TACAGTTTCTAACCTCTGAATTTTTTACTGATATTTAGACGGTCTTTCCTTATCCTCAGACCGTCAAACCTTTACCTACGT  
 ACTTGGCGCGCAGTACGTTTCAATTTCAAAATGGAATAGAC

## 40 SEQ. ID NO:21

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the Lipo02 gene from *Neisseria meningitidis* (serogroup B)**

TTATCTTGGTGCAAACTTTGTGCGGGTTCGACTGGCTACGGCTTTGGGTTTGGACCCGCTCATCGGTCTGATTACCGGT  
 TCGGTGTGCTGACGGGCGGACACGGTACGTCAGGTGCGTGGGGACCTAATTTTGAACGCAATACGGCTTGGTTCGGCGC  
 45 AACCGGTTTGGGTATGTCATCGGCTACTTTCGGGCTGGTGTTCGGCGGCCCTGATCGGCGGGCCGGTTGCGCGCCGCTGA  
 TCAACAAAATGGGCCGCAACCGGTTGAAAACAAAAACAGGATCAGGACGACAACGCGGACGACGTGTTGAGCAGGGCA  
 AAACGCACCCGCTGATTACGGCGGAATCTGCCGTTGAAACGCTTGCCATGTTTGGCGCGTGTGTTGGCGTTTGGCGAGAT  
 TATGGACGGCTTCGACAAAGAATATCTGTTGACCTGCCCAAATTCGTGTGGTGTCTGTTTGGCGGCGTGGTTCATCCGA  
 ACATCCTCACTGCCGATTTCAAGGTCAATATGTTGACCGCGCCATCGATGTGTTGCGCAATGCTTCGCTTTTCGCTTTTC  
 50 TTGGCAATGGCGTTGCTGAATTTGAAACTGTGGGAGCTGACCGGTTTGGCGGGGCTGTAAACCGTGATTCTTGCCGTACA  
 AACCGTGGTGTGTTTGTACGCGACTTTTGTACCTATGTCTTTATGGGGCGGACTATGATGCGGCAGTATTGGCTG  
 CCGGCCATTGCGTTTTCGGCTTGGGTGCAACGCCGACGGCGGTGGCAAATATGCAGTCCGTCACGCATACCTTCGGCGCG  
 TCGCATAAGGCGTTTTTGAATGTGCTATGGTGGCGCGTCTTTCGTCGATTTGATTAATGCCGCGATTCTCACCGGTTT  
 55 TGTGAATTTCTTAAAGGCTGATTTTCCGCCCTTCCGACAAAGCACCTGCAAGGTTTACCGCCTGCAGGTGCTTTTGCTA  
 TGATAGCCGCTATCGGTCTGCACCGTTTGAAGGAACATC

## SEQ. ID NO:22

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the Tbp2 gene from *Neisseria meningitidis* (serogroup B)**

60 CCTACTCCACCGATTCCAATATGCTCGGCGCGACCCACGAAGCCAAAGACTTGGAATTTTTGAACTCGGGCATCAAATC  
 GTCAAACCCATTATGGGCGTTGCCCTTTGGGACGAAAACGTTGAAGTCAGCCCCGAAGAAGTCAGCGTGCCTTTGAAGA  
 AGGCGTGCCGTTGCACTGAACGGCAAAGAATACGCCGACCCCGTCGAATCTTCTCGAAGCCAACCGCATCGGCGGCC  
 GCCACGGCTTGGGTATGAGCGACCAAAATCGAAAACCGCATCGAAGCCAAATCGCGCGGCATCTACGAAGCCCCGGGT  
 ATGGCGTTGTTCCACATCGCCTACGAACGCTTGGTGACCGGCATCCACAACGAAGACACCATCGAACAATACCGCATCAA  
 65 CGGCCCTGCGCCTCGGCCGTTTGGCTTACCAAGGCCGCTGGTTCGACAGCCAAGCCTTGATGTTGCGCGAAACCGCCCAAC  
 GCTGGGTGCGCAAAGCCGTTACCGGCGAAGTTACCCTCGAAGTGCAGCGCGGCAACGACTACTCGATTCTGAACACCGAA  
 TCGCCCAACCTGACCTACCAACCCGAACGCTGAGTATGAAAAAGTCGAAGGTGCGGCGTTTACCCCGCTCGACCGCAT  
 CGGACAGCTCACGATGCGCAACCTCGACATACCGACACCCGCGCCAAACTGGGCATCTACTCGCAAAGCGGTTTGCTGT  
 CGCTGGGCGAAGGCTCGGTATTACCGCAGTTGGGCAATAAGAAATAAGGTTTGTGTTTGCATCATTTAGCAACTTAAGG  
 70 GGTGCTCTGAAAAGATGATCCCTTATGTTAAAGGAATCCTATGAAAGAATACAAAGTCGTCAATTTATCAGGAAAGCCAG  
 TTGTCAGCCTGTTTTTCGGCGCGGCAAAGGTCAACCCCGTCAATTTACGCGCGTCTCTCAACAAACAAACCCCGGAAG  
 GCTGGCGGGTCGAGACCTTTGCAATAACATAGGTTACTAA



SEQ. ID NO:23

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the PorA gene from *Neisseria meningitidis* (serogroup B)**

5 GAATGACAATTCTATAAGTTTCCCGAAATTCACATAACCGAAACCTGACAATAACCGTAGCAACTGAACCGTCATTCCC  
GCAAAAGCGGGAATCCAGTCCGTTTCAGTTTCGGTCATTTCCGATAAATGCCTGTTGCTTTTCATTTCTAGATTCCCACTT  
TCGTGGGAATGACGGCGGAAGGGTTTTGGTTTTTCCGATAAATCTTGAGGCATTGAAATTCCAAATTCCTGCTGCGC  
10 GGGAAATGACGGCTGCAGATGCCCGACGGTCTTTATAGTGGATTAACAAAAATCAGGACAAGGCGACGAGCTGCAGACAGT  
ACAGATAGTACGGAACCGATTCACTTAGTGCTTCAGTATCTTAGAGAAATCGTTCTCTTTGAGCTAAGGCGAGGCAACGTC  
GTACTGGTTTTTTGTTTCATCCACTATATATGACACGGAAACGCCGCCGTCCAAACCATGCCGTCTGAAGAAAACCTACACA  
GATACCGCCGCTTATATTACAATCGCCGCCCGCTGGTTCGAAAACCTCCACACTAAAAAACTAAGGAAACCTATGTCC  
CGCAACAACGAAGAGCTGCAAGGTATCTCGCTTTTGGGTAATCAAAAAACCCAATATCCGGCCGAATACGCGCCCGAAAT  
15 TTTGGAAGCGTTCGACAACAAACATCCCGACAACGACTATTTTCGTCAAATTCGTCTGCCCAGAGTTCACCAGCCTCTGCC  
CCATGACCGGGCAGCCCGACTTCGCCACCATCGTCATCCGCTACATTCCGCACATCAAAATGGTGGAAAGCAAATCCCTG  
AAACTCTACCTCTTCAGCTTCCGCAACCACGGCGATTTCATGAAGACTGCGTCAACATCATCATGAAAGACCTCATTGC  
CCTGATGGATCCGAAATACATCGAAGTATTCGGCGAGTTCACACCGCGCGGGCGGCATCGCCATTCTCTTCGCCAATT  
ACGGCAAAGCAGGCACCGAGTTTGAAGCATTGGCGCGTAA

SEQ. ID NO:24

***Neisseria meningitidis* (serogroup B) PorA Promoter Region**

20 GATATCGAGGTCTGCGCTTGAATTGTGTTGTAGAAACACAACGTTTTTGAAAAATAAGCTATTGTTTTATATCAAAATA  
TAATCATTTTTTAAATAAAGGTTGCGGCATTTATCAGATATTTGTCTGAAAAATGGTTTTTTGCGGGGGGGGGGTATA  
ATTGAAGACGTATCGGGTGTTCGCCGATGTTTTTAGGTTTTTATCAAATTTACAAAAGGAAGCCCAT

25

SEQ. ID NO:25

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the PorB gene from *Neisseria meningitidis* (serogroup A)**

30 gttttctgtttttgaggggaatgacgggatgtaggttcgtagaagaatgacgggatataggtttccgtgaggatggattcgtc  
attccccgcgcaggcggaatctagaacgtggaatctaagaaaccgttttatccgataagtttccgtgaggacaagtttgg  
attccccgcctgacgggaatgacgggatgttaggtttctaatgttttctgtttttgaggggaatgacgggatgtagg  
ttcgtaggaatgacgggatataggtttccgtgaggatggattcgtcattccccgcgcaggcggaatctagacctagaac  
aacagcaatattcaaagattatctgaaagtccgagattctagattccccgcctgagcgggaatgacgaaaagtggcgggaa  
tgacggttagcgttgcctcgccttagctcaaagagaacgattctctaaggtgctgaagcaccaagtgaatcgttccgta  
35 ctatttgtactgtctgcggttcgtcgccttgctcgtatttttgttaatccactatctcctgcccaggggcggttttg  
catccgcccgttccgaaagaaccgcgtgtgctgttttttgcggtctttataacccccggtttgcaatgccctccaatacc  
ctcccgagtaagtgttgtaaaaaatgcaaatcttaaaaaatttaaataaccatattgttataaaacaaaaataaccataat  
atctctatccgtccttcaaatgcacatcgaaattccacacaaaaacaggcagaagtttgttttttcagacaggaacatct  
atagtttcagacatgtaatcgccgagcccctcgccggttaaatgcaaagctaagcggccttggaagcccggcctgcttaaa  
40 tttcttaacaaaaaaggaatacagcaatgaaaaaatccctgattgccctgactttggcagcccttccctgttgagcaat  
ggctgacgttaccctgtacggcaccatcaaaaccggcgta

SEQ. ID NO:26

***Neisseria meningitidis* (serogroup B) PorB Promoter Region**

45 GTTTTCTGTTTTTGAGGGAATGACGGGATGTAGGTTCTGAAGAATGACGGGATATAGGTTTCCGTGCGGATGGATTCTGTC  
ATTCCCGCGCAGGCGGGAATCTAGAACGTGGAATCTAAGAAACCGTTTTATCCGATAAGTTTTCCGTGCGGACAAGTTTG  
GATTCCCGCCTGCGCGGGAATGACGGGATTTTAGGTTTCTAATTTTGGTTTTCTGTTTTTGAGGGAATGACGGGATGTAG  
GTTCTAGGAATGACGGGATATAGGTTTCCGTGCGGATGGATTCTGTCATTCCCGCGCAGGCGGGAATCCAGACCTTAGAA  
CAACAGCAATATTCAAAGATTATCTGAAAGTCCGAGATTCTAGATTCCCGCCTGAGCGGGAATGACGAAAAGTGGCGGGA  
50 ATGACGGTTAGCGTTGCCTCGCCTTAGCTCAAAGAGAACGATTCTCTAAGGTGCTGAAGCACTAAGTGAATCGGTTCCGT  
ACTATTTGTACTGTCTGCGGCTTCGTGCGCTTGTCTGATTTTTGTAAATCCACTAT

SEQ. ID NO:27

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the siaABC gene from *Neisseria meningitidis* (serogroup B)**

55 ATACGGCCAATGGCTTCAGAAAGCGATAAGCCTCTGGCTGAAAAACCGATTCTTGTGTTCTCCCCACCGCACCCATAGA  
CGTAAAGGTATAGGGATTGGTAATCATGGTAACCACATCACCGCGACGCAGCAAAATATTTTGTGCGGGATTGCAACTA  
AATCTTCCAAGGCAACAGTTCGTACTACATTGCCACGTGTCAGCTGCACATTCTGACATTGCGGTTGAACCA  
CCTACCGCAGCCACCGCATCCAACACACGCTCACCGGCTGCCGTGAGCGGCATACGCACACTATTCCCAGCACGAATCAC  
60 CGACACATTGCGCCGATTATTCTGCACCAAACGCACCATCACTTGTGGCTGATTGGCCATTTTTTTTTCAGGCGGCCTTTAA  
TAATTTCTTGAACCTGACCAGGCGTTTACCGACCACCGAAATATCGCCAACAAACGGCACAGAAACCGTACCACGTGCC  
GTGACCACTGCTCTGGCAACTTAGTTGATGCGCATCAACCGAGCCCATCGAAGAAAGGCCACCACCAACAATACTGC  
CGGCGCGCTTCCCAAATCATAATATCCAATACACCAATATTTAGCGTACCAGCCGAAGCATAACCAATCGCCAAACT  
GAGTGAATGACTGATTTATCTGAGCCTTATATAATAAAGTACGCAACCGTATGATTACATCAATCAGCTCCACTTCAGGA  
65 ATTTGAACCTCAGATTGTTGCCCTAAAGAGACAATTTTTCGCTGGGGCCTGATGAAGGAATCGCAGAGCATCCTAC  
AATTAACCTTCCACACAATAATAACTGCGTGACGAATATAAAATTTCACTTAAACACAAGCCAAATCCTAATATAAT  
TATAAATGGCCTAATTATAGCACTTAATCGAAATAAATTTATGAGTACGTAGAGTATAATTAGTATTCTTCTTTCCAAC  
TCCTTATACTTATATATATACTTATAGATTCTAAAATC

70 SEQ. ID NO:28



**Nucleotide sequence of DNA region (1000 bp ) up-stream from the lgt gene from *Neisseria meningitidis* (serogroup B)**

5 GCCAAAGCATTGGGCGCGGATGCCGCCGCTGCCGAACGCGCCGCGCTCTTGCCAAAGCCGACTTGGTAACCGAAATGGT  
CGGCGAGTTCCCCGAAC TGCAAGGCACGATGGGCAAATACTATGCCTGTTTGGACGGCGAAACCGAAGAAATTGCCGAAG  
CCGTCGAGCAGCACTATCAGCCGCGTTTGGCCGGCGACAAGCTGCCCGAAAGCAAATTGCCGCCGCGCGTGGCACTGGCC  
10 GACAAACTAGAAACCTTGGTCGGCATTGGGGCATCGGTCTGATTCCGACCGGCGACAAAGACCCCTACGCCCTGCGCCG  
CGCTGCCTTGGGTATTTGCGTATGCTGATGCAGTATGGTTTGGACGTGAACGAACGATTCAGACGGCATTGACAGCT  
TCCCCAAAGTTTGTCTAACGAAAAAACGCCGTCTGAAACCGCCGACTTTATGCAGGCGCGCCTTGCCGTGTTGCTGCAA  
AACGATTATCCGCAAGACATCGTTGCCGCCGTACTCGCCAAACAGCCGCGCGCTTGGACGATTTGACCGCCAAACTGCA  
15 GGCCGTTGCCGCGTTCAAACAAC TGCCCGAAGCCGCGCGCTCGCCGCCCAACAAACGCGTGCAAAACCTGCTGAAAA  
AAGCCGATGCCGAGTTGGGCGCGGTTAACGAAAGCCTGTTGCAACAGGACGAAGAAAAAGCCCTCTTTGCCGCCGCGCAA  
GGCTTGACGCCGAAAAATCGCCGCCGCGCTCGCCGAAGGCAATTTCCAAACCGCCTTGTCGAACTGGCTTCCGTCAAACC  
GCAAGTCGATGCATTCTTTGACGGCGTGATGGTAATGGCGGAAGATGCCGCGTAAACAAAACCGCCTGAACCTGCTGA  
ACCGCTTGGCAGAGCAAATGAACGCGGTAGCCGACATCGCGCTTTGGGCGAGTAACCGTTGTACAGTCCAAATGCCGTC  
15 TGAAGCCTTCAGACGGCATCGTGCTATCGGGAGAATAAA

SEQ. ID NO:29

**Nucleotide sequence of DNA region (1000 bp) up-stream from the TbpB gene from *Neisseria meningitidis* (strain MC58)**

20 GAACGAACCGGATTCCCCTTTCTGTTGGGAATGACGAATTTTCTGTTTCTGTTTTTGTGAAAAATAAT  
GGGATTTTCTGTTGGGTATTTACCGGAAAAACAGAAACCGCTCCGCCGTCATTCCCGCGCAGGCGGGAATCTAGGTC  
TGTCGGTGCGGAACTTATCGGATAAAACGGTTTCTTGAGATTTTCTGTCCTGGATTCCCCTTTCTGTTGGGAATGACGCG  
AACAGAAACCGCTCCGCCGTCATTCCCGCGCAGGCGGGAATCTAGACATTCAATGCTAAGGCAATTTATCGGGAATGACT  
25 GAAACTCAAAAACTGGATTCCCACTTTCTGTTGGGAATGACGTGGTGACGGTTTCCGTATGGATGGATTCCGTCAATCCCGC  
GCAGGCGGGAATCTAGACCTTCAATACTAAGGCAATTTATCGGAAATGACTGAAACTCGAAAACTGGATTCCCCTTTT  
GTGGGAATGACGCGATTAGAGTTTCAAAATTTATTCTAAATAGCTGAAACTCAACACACTGGATTCCCGCCTGCGCGGGA  
ATGACGAAGTGGAAGTTACCCGAACTTAAACAAGCGAAACCGAAGCAACTGGATTCCCCTTTCTGTTGGGAATGACGGA  
ATGTAGGTTCTGTTGGGAATGACGCGCGGAGCGGTTTCTGCTTTTCCAATAAATGACCCCACTTAAATCCCCTCATTCCC  
30 GCGCAGGCGGGAATCTAGGTCGTGTCGGTTCGGAACTTATCGGGTAAACGGTTTCTTGAGATTTTGCCTCCTGGATTCC  
CACTTTCTGTTGGGAATGACGGAATGTAGGTTCTGTTGGGAATGACGGGATATAGGTTTCCGTGCGGACGCGTTCCGATTCTATG  
ACTGCGCGGGAATGACGGGATTTTGGTGATTCCCTAAAAAATAAAAAAGTATTTGCAAATTTGTTAAAAATAAATAAA  
ATAATAATCCTTATCATTCTTTAATTGAATTGGATTTATT

SEQ. ID NO:30

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the opc gene from *Neisseria meningitidis* (serogroup A)**

35 CAAAGGCTACGACAGTGCGGAAACCGGCAACATCTGGAAGAACATCAGTTGTTGGACGGCATTATGCGCAAAGCCTGCC  
GCAACCGTCCGCTGTGCGGAAACGCAAAACGCAACCGGTATTTGTGCAAGACCCGTATATAGTGGATTAAATTTAAAT  
CAGGACAAGGCGACGAAGCCGACAGTACAAATAGTACGGCAAGGCGAGGCAACGCCGTACTGGTTTAAATTTAATCC  
40 ACTATATGTGGTGAACAGAGCTTCGGTACGCTGCACCGTAAATTCGGCTATGCGCGGGCAGCCTATTTCCGACTGATTA  
AAGTGAGTGCGCAAAGCCATCTGAAGGCGATGTGTTTGAACCTGTGAAAGCCGCCAACAAAGCTAAGTGCGCCCGCTGCC  
GCCTAAAAGGAGACCGGATGCCTGATTATCGGGTATCCGGGGAGGGTTAAGGGGGTATTTGGGTAAAATTAGGAGGTATT  
TGGGGCGAAAAATAGACGAAAACCTGTGTTTGGGTTTCCGGCTGTGCGGGAGGGAAGGAATTTGCAAAGATCTCATCCTGT  
45 TATTTTCACAAAAACAGAAAAACCAAAACCGGACGAACTAGTATTCCTCCGCTTTCCGCGGGAATGACGGCAGAGTGTTT  
GCGGCAGGAATCTATCGGAAATAACGAAACCGGACGAACTAGTATTCCTCCGCTTTCCGCGGGAATGACGGCAGAGTGTTT  
CAGTTGCTCCCGATAAAATGCCGCCATCTCAAGTCTCGTCATTCCCTTAAACAGAAAAACCGAAATCAGAAACCTAAATTT  
TCGTCAATCCCATAAAAACAGAAAAACCAAGTGAGAATAACAATTCGTTGTAAACAAATAACTATTTGTTAATTTTATT  
AATATATGTAAATCCCCCCCCCCCCCCCCCGAAAGCTTAAGAATATAATTGTAAGCGTAACGATTATTACGTTATGTT  
50 ACCATATCCGACTACAATCCAAATTTTGGAGATTTTAACT

SEQ. ID NO:31

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the siaD gene from *Neisseria meningitidis* (serogroup B)**

55 ATAATGCAGGCGCTGAAGTTGTTAAACATCAAACACACATCGTTGAAGACGAAATGTCTGATGAGGCCAAACAAGTCATT  
CCAGGCAATGCAGATGTCTCTATTTATGAAATTATGGAACGTTGCGCCCTGAATGAAGAAGATGAGATTAAATTTAAAGA  
ATACGTAGAGAGTAAGGGTATGATTTTATCAGTACTCCTTTCTCTCGTGCAGCTGCTTTACGATTACAACGTATGGATA  
TTCCAGCATATAAAATCGGCTCTGGCGAATGTAATAACTACCCATTAATTAAGCTGGTGCCCTCTTTTGGTAAGCCTATT  
ATCTCTCTACCGGCATGAATTCTATTGAAAGCATCAAAAAGTCGGTAGAAATTATTCGAGAAGCAGGGGTACCTTATGC  
60 TTTGCTTCACTGTACCAACATCTACCCAACCCCTTACGAAGATGTTTCGATTGGGTGGTATGAACGATTTATCTGAAGCCT  
TTCCAGACGCAATCATTGGCCTGTCTGACCATACCTTAGATAACTATGCTTGTCTAGGAGCAGTAGCTTTAGGCGGTTTCG  
ATTTTAGAGCGTCACTTTACTGACCGAATGGATCGCCAGGTCCGGATATTGCTCTATGCTCTATGAATCCGGTACTTTTAA  
AGAGCTCAAGCAAGGCGCTCATGCTTTAAATTTGGCACGCGCGGCAAAAAGACACGATTATCGCGGGAGAAAAGCCAA  
CTAAAGATTTGCGCTTTGCATCTGTCTAGCAGATAAAGACATTAAAAAAGGAGAACTGTTGTCCGGAGATAACCTATGG  
65 GTTAAACGCCAGGCAATGGAGACTTCAGCGTCAACGAATATGAAACATTATTTGGTAAGGTCGCTGCTGCAATATTTCG  
CAAAGGTGCTCAAATCAAAAAACTGATATTGAATAATGCTTATTAACCTAGTTACTTTATTAACAGAGGATTGGCTATT  
ACATATAGCTAATTCTCATTAATTTTAAAGAGATACAATA

SEQ. ID NO:32

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the ctrA gene from *Neisseria meningitidis* (serogroup B)**

70 ATACCTGCACTTGAGTTGCCGACCATAAATTTAGCATGTTTCAATAAGACTAAAAAATATTCAAATCGAATGGAAGGAAA

TGAATAAAATTTATCAGATTGATATTTTAATAATTCTTGCAGAATACTTTAGTGCCAGTGTCATTATTAGGGTAGATGC  
 TAATGATATTTTGGCCACTTAATTCTAATGCTTTGAAATATTGGGCCGCATATTGTGGCATTAAATGTGCTTCTGTAGTC  
 ACGGGGTGAAACATAGAAATACCATAATTTTCGTATGGTAAACCGTAATATTCTTTGACTTCTTCTAAGGATGGGAGGGT  
 5 GGAAGAGGCCATAACATCTAAATCGGGGGAGCCGATGATGTGAATATGCTTTCTTTTCTCCATTTCGACTAGGCGAG  
 TGACAGCTTGTTTCATTGCTACCAAGTGGATATGAGAAAGTTTACTAATAGAATGACGAATGGAGTCATCTACTGTACCA  
 GATAGTTCACCACCTTCGATATGGCAAATAAACGGCTGCTTAATGCACCTACAGCTGCGCCTGCTAGTGCTTCTAAACG  
 GTCGCCGTGAATCATGACCATATCAGGTTCAATTTTCATCAGATAGACGAGAGATAAACGTAATGGTATTGCCTAAAACGG  
 CACCCATTGGTTTACCTTGGATTTGATTGAAAACAGATATGTATGTTGATAGTTTTCTCGAGTTACTTCCTTGTAGGTT  
 10 CTGCCATATGTTTTTCATCATATGCATACCAGTTACAATCAAATGCAATTCAGGTCTGGGTGATTTTCAATATAGGCTAA  
 TAAAGTTTTAGCTTGCCGAAGTCGGCTCTGGTACCTGTAATGCAAAGAATTCTTTTCATGATTTTAGAATCTATAAGTA  
 TATATATATAAGTATAAGGAAGTTGGAAAGAAGATACTAATTATACTCTACGTACTCATAAATTTATTTTCGATTAAAGTG  
 CTATAATTAGGCCATTATAATTATATTAGGATTTGGCTT

SEQ. ID NO:33

15 **Nucleotide sequence of DNA region (1000 bp ) up-stream from the lgtF gene from *Neisseria meningitidis* (serogroup A)**

TCTTTTTTCGGACTGAAAGGACGCATCATCCCGACATCGAGCGCGTGTTCGTCCGGCAGCCAAGGCATAGGTTATGCCTAC  
 GAAGCCATCAAATACGGTCTGACCGATATGATGCTGGCGGGCGGAGGCCAAGAATTTTCCCGTCCGAAGTGTATGTTTT  
 CGACTCGCTTTATGCCGCCAGCCGCCGCAACGGCGAACCAGGAAAAACCCCGCGCCCATACGACGCGAACCAGCGAGGGC  
 20 TGGTCATCGCGCAAGGCGCGGGGATTTTCGTGCTGGAAGAATTGGAACACGCCAAACGGCGCGGTGCGATAATTTACGCC  
 GAACCTCGTCGGCTACGGAGCCAACAGCGATGCTTACCATATTTCCACGCCCGCCCGACGCGCAAGGCGCAATCCTTGC  
 CTTTCAGACGGCATTTGCAACACGCAGACCTTGCGCCCGAAGACATCGGCTGGATTAATCTGCACGGCACCAGGACGCACC  
 ACAACGACAGTATGGAAGCCGCGCGCTTGACGCGGTTTTCGGCAACAATACGCCCTGCACGTCCACCAAGCCGCAAAAC  
 GGACACACGCTGGGCGCGGGCGGCGCAATCGAAGCCGCGTTCGCGTGGGGCATTTGCTGACCGGAAAAGCAATCCCGAAGG  
 25 GAACTTCCGCCCCAGCTTTGGGACGGGCGAAGCATCCCGACCTTCCCGCCATCAACCTGACCGGCAGCGGCAGCCGCT  
 GGGAAACCGAAAAACGCATTGCCGCCAGCTCGTCTGCTTTGCCCTTCGGAGGAAGCAACTGCGTTTTACTCATCGGATGAAAT  
 AAGTTTGTCAATCCCACCGCTATGCTATACAATACGCGCCTACTCTTGATGGGTCTGTAGCTCAGGGGTAGAGCAGGGG  
 ACTCATAATCCCTTGGTCTGGGTTCGAGCCCCACCGACCCACCAATTCCCAAGCCCGGACGTATGTTTGGGCTTTTTT  
 30 GCGCCCTGTGAAACCAAATGCTTTGAGAAACCTTGATA

SEQ. ID NO:34

35 **Nucleotide sequence of DNA region (1000 bp ) up-stream from the lgtB gene from *Neisseria meningitidis* (serogroup B)**

TAGAAAAATATTTTCGCCCAATCATTAGCCGCCGTGCTGAATCAGACTTGGCGCAACTTGGAGATTTTGATTGTGATGAC  
 GGCTCGACAGACGGTACGCTTGCCATTGCCAAGGATTTTCAAAGCGGGACAGCCGTATCAAATCCTTGACACAAGCTCA  
 40 AAATTCGGCCTGATTCCTCTTTAAACATCGGGCTGGACGAATTGGCAAAGTCAGGAATGGGGGAATATATTGCACGCA  
 CCGATGCCGACGATATTGCCGCCCGCCCGACTGGATTGAGAAATCGTGGGCGAGATGGAAAAAGACCGCAGCATCATCGCG  
 ATGGGCGCGTGGCTGGAAGTTTTGTCGGAAGAAAAGGACGGCAACCGGCTGGCGCGGCATCACAGGCACGGCAAAATTTG  
 GAAAAAGCCGACCCGGCAGCAAGATATTGCCGACTTTTCCCTTTTCGGCAACCCCATACACAACAACAGATGATTATGA  
 GGCGCAGCGTCATTGACGGCGGTTTGCGTTACAACACCGAGCGGGATTGGGCGGAAGATTACCAATTTTGGTACGATGTC  
 AGCAAATTGGGCAGGCTGGCTTATTATCCCGAAGCCTTGGTCAAATACCGCCTTCACGCCAATCAGGTTTCATCCAAATA  
 CAGCATCCGCCAACACGAAATCGCGCAAGGCATCCAAAAACCGCCAGAAACGATTTTTTGCAGTCTATGGGTTTTAAAA  
 CCCGGTTTCGACAGCCTTGAATACCGCCAAATAAAAGCAGTAGCGTATGAATTGCTGGAGAAACATTTGCCGGAAGAAGAT  
 45 TTTGAACGCGCCCGCGGTTTTTGTACCAATGCTTCAAACGGACGGACACGCTGCCCGCCGGCGCGTGGCTGGATTTTGC  
 GGCAGACGGCAGGATGCGGCGGCTGTTTACCTTGAGGCAATACTTCGGCATTTTGACCGATTGCTGAAAAACCGTTGAA  
 AACGCGCGCTTTATCCAACAGACAAAAACAGGATAAATT

SEQ. ID NO:35

50 **Nucleotide sequence of DNA region (1000 bp ) up-stream from the lgt gene from *Neisseria meningitidis* (serogroup B)**

GCGCAGCGCTTTTTCTTCATCGGTTTGAGGGTGGCGAGGATAATCGGGGACGGCAAAGCCTTTAGACTGCAATTCTTTAA  
 TCGCGGCGGTTCAGTTGAGGTACGGATGCGCTGATGTCGGCAGTTTGATTACGTTTGCATCGGGCTGTTTCACCAGTTCG  
 CCCAATTCGGCAAGCGCGTTCGGGTACGCGCTCGGCTTCGGTCAGATATTGGGGGAATGCCGCCAAAATACGGCCGGACAG  
 55 GGAAATGTCGGCAGTTTTTGACATCAATATCGGCGTGGCGGGCAAACGCTGCACAATCGGCAGCAGCGATTGGGTTCGCCA  
 GCGCGGGGGCTTCGTGCGTATGGGTATAAACAATGGTGGATTTTTGAGTCATAGGATTATTCTCTTGTAGGTTGGTTTTT  
 TCTTTTGAACACATTGCGCGGGGAATGTGCGCGGCTATTATGGCATATTTTGGCGGCTTTGTTTCGCGCTTTGTTTCGATC  
 TTGGCGTGTGTTGAACGCGGCAGCGTGAAAGGAAGGGGGAATGGTTTTCCCGCGTTTGGCGGGCGGTGTCGGAGGTGCTGT  
 GCCTGATGTGCGGCGGCATATTTTCGGTGAAATTGATTTTATAGTGGTTTAAATTTAAACCAGTACAGCGTTGCCCTCGCC  
 TTGTGCTACTATCTGTACTGTCTGCGGCTTCGTTGCCCTGTCCTGATTTAAATTTAAACCACTATAATATTCCGGTAACTG  
 60 TCGGAATATCTGCTAAATTCGCGATTTTTCCGCCCTCGGGACACTCGGGGCGTATGTTTAATTTGTCGGAATGGAGTTTT  
 AGGGAT

SEQ. ID NO:36

65 **Nucleotide sequence of DNA region (1000 bp ) up-stream from the msbB gene from *Neisseria meningitidis* (serogroup B)**

GCCCGACGGCGAACAGACACGTCGTAATCAACCGCTTGGACAGTACGGCGGCGCAATACGACATGCTTGCAGGTTATC  
 TTGAAAGACTTGCCGGAACAAACCGACCGTTGGGCGTGCGCCTACCGCCAAAATGCCGTCTGAACACCCGATTATCCTTTT  
 GAAAGCGGATTTATGCCCCATACCCTTCCCGATATTTCCCAATGTATCAGACAAAATTTGGAACAATATTTCAAAGACCT  
 70 GAACGGTACCGAACCTTGGCGGTGTACGATATGGTCTTCATCAGGTGGAAAAACCGCTGCTGGTGTGCGTGATGGAAC  
 AATGCGGCGGCAACCAAGTCCAAAGCCTCCGTCATGTTGGGACTGAACCGCAATACCTTTCGCTAAAAAACTGATTCAACAC  
 GGTTTTGCTGTGAATATGTCGGCAACCGTCCGTATCTTGGGTATTGACCCGGGCGAGTCGCGTAACGGGTTTTCCGTTGTCATC  
 GATGTGAGGGGGCGGATCATTTTTACGTCGCTCCGGCTGCATCAAACGCGCTGCCGATGCGCCTCTGGCAGACAGGAT  
 TGCCGTGATTGTGCGGCATATCGGCGAAGTCGTTACCGTTTACAAGCCTCAACAGGCGGCGAGTGAACAGGTGTTTCGTC



ACGTCAATCCGGCATCGACGCTGATGCTCGGTCAGGCTAGGGGCGCGGCATTGGCGGCATTGGTCAGCCATAAGCTGCCC  
 GTTTCGGAATACACGGCCTTGCAGGTCAAACAGGCGGTAGTCGGCAAGGGCAAGGCGGCAAAAGAACAGGTGCAGCATAT  
 GGTGGTGCAGATGCTGGGGCTTTCGGGAACGCCGAGGANTGGCGCGGACGGTCTTGCCGTCGCGCTGACCCACGCCTT  
 5 ACGCAACCACGGGCTTGCCGCCAACTCAATCCTTCGGGGATGCAGGTCAAGCGCGGCAGGTTTCAATAGTTTCAGACGG  
 CATTGTATTTTGCCGTCTGAAAAGAAAATGTGTATCGAG

SEQ. ID NO:37

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the htrB gene  
 from *Neisseria meningitidis* (serogroup B)**

10 CCGCCAAGCGTTTCCCCCTTTGTGTCGGGCTTAACATTTGCTTTGTACGGCAGACTTTTTCCCTTCATAACGCCGCTTTCC  
 GAAAAGACGATGGTAGGCGCGACGTAATTCTCAACCCTTAAGGTACGGTTGGACGAAAAGTTTCCCTTTTCATTCCACCT  
 GCCAATTTTTCGGCTACACCGAGTGGTCTCGTTAGGTTTGGGCGAACTACGCCCTTAAAAAACGGACATTCTTTGCATG  
 CCCGTCTCTAAGGTTTACGGTAAGTTTACCTTATAAAGAGTTGACTTACCATACTTATCCCTTTAAACGATATAAAG  
 15 GGCGACAGCTGTAATACAAGTATGTTGTACGGCAGACTTCTTCTACCAAACAAAAAGTTCCCTTTAGAGTTACTCGCTTA  
 TAGACAAATGAAGGCTTAGCCATAGGCTTCCGGTAGGCCTATTTCAACGGCTGGTTCACAGGCTACGCTAAAACCTACGG  
 TAGAACCGCGTTCTGGGGTTTCGCGCACAGCGGCGTCTTTGGAACCAGTTGTGTCCGAACACGCATAACCGCCCGCTTTA  
 ATGGTGGTGGCGGGTTACCTGATGTAGTTTCAGCGTGGCTTTGGTAGTTTTCGTAGCCGATGTTGAGGAGGCTCGACC  
 CGAACTACGGTTGCCGACGCGCCAGCCGCACATGATGCTGGTCTGTAGAGGCTGTAGCGGGTTCCGCACCTTGCTTCCG  
 20 CTTCCGTAACCTGAACCTGGTTCCGCGACCGCTGGTTCCAAACTACAAGCCGATACGCGACGCTGCTTTGGGGCTGGGACTA  
 CGGCAACGGTAGATAATGTCGGTGGCGGACTACGTCGCAGTTTTCGCTTAATGCGTTTCTGCCGGAGGACGGAACCGACG  
 CAGGGCTGCGTTTTTCGGGTTGACTGGCACCAATGCTATCGCTTAGGCCGTTTCATTTTGCGTAACTATGGCAGCAGGAG  
 AGATACGTTGTGCTGGGCCCTTAGCCAATACTTCTCAACT

SEQ. ID NO:38

25 **Nucleotide sequence of DNA region (1000 bp ) up-stream from the MltA gene  
 from *Neisseria meningitidis* (serogroup B)**

30 CACAAAAACCAAGTTATGACGGGAATAAGGTACAGCAGCCAAACCAAGGCCCTCGCCCTGC  
 GTCGGATGGTCGGTATAGCCGAAAAATCCGCCGAGCAGCAGCCCAACGGGCTGTCTTCG  
 TGCAAATATTTTATGAGTCGAACACAATGTCCTGAAGCGGTTCCAAATGCCTGCTTCG  
 TGCAGCGCACGCAGCGAACCAGGCAAGCAGACCAGCGGCAACGATAATCAGAAACGCCCT  
 GTCCAACGGAAAACTTCGCCAGATTTCAGGCGCATCCACCTGATAAATCAACGCGCCA  
 ATCAGCGCGGCAGCCAAAACCCCGCTACCGCACCGCGCGGCATCTGCCACGTCGGGCTC  
 TGTTTGAATACGGCAAGCAGGAAAAAACGCTCTCCAAACCTTCGCGCGCCACGGCAAGA  
 AACGCCATACCGACCAAGGCCCTTCTGACCGCTGCCACGGTTCAAAGCCGCTGCACA  
 35 GAATCCTGAAGCTGCCGCTTTCATCGACCGGGCTTTTTTTCATCCATAAAATCATATAA  
 GTCAGCATCGCGACAGCAACCAGGATAATGCCGACGACGAACCTCTGCTGCTTCTGG  
 GGAATCTCGCCCGTTGCCGAATGGATTCCGTACCCAGCCCCAACACATCAAAGAAGCA  
 AGAACAACCCCGAACCAGACCTTAGGCATCAGTTTGAATGTCCGGACTGTTTCAGAAAA  
 CCGGCAACGATGCCGACGATGAGCGCGGCTTCGATACCCTCGCGCAACATAATTAAAAAA  
 40 GCGACCAGCATAAACCGCAACGAACAAGGATGATGAATAATATATATTCGGAATATTTTC  
 ATTGCTTGTAATAACAAATGCAAGTTATTTTTATCTGCAGTACCGCGCGCGGAAAGTTC  
 CGCAGCTGCAGCTGCGCCCTGTGTTAAATCCCTCTCCACGGCTGCCGCAACGCCGCC  
 GAAACCATCTTTCTTATTACTGCCGGCAACATTGTCCATT

45 SEQ. ID NO:39

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the ompCD gene  
 from *Moraxella catarrhalis***

50 GCTGATTTGTGAGCAAGCGGGCGCATCAGGGATTACCTTGCATTTGCGAGAAGATCGTCC  
 ACATATTCAAGATGAAGATGTTTATGAATTGATTGGGCAATTGACAACACGCATGAATCT  
 TGAGATGGCAGTCACTGATGAGATGCTAAATATTGCCCTAAAGGTACGACCAGCATGGGT  
 GTGTTTAGTACCAGAAAAACGCCAAGAGCTGACTACAGAAGGTGGGCTTGATATCGCCAA  
 TTTATCAAAATATTCAAGCATTATACACAGTCTTCAGCAGGCGGATATTAAGGTTTCTTT  
 ATTCATCGATCCAGATCCGCATCAAAATTGATGCTGCAATTGCTTTGGGTGCTGATGCGAT  
 TGAGCTGCATACGGGAGCTTATGCTCAAGCGACTTTACAAAATAATCAAAGCTTGTTGA  
 55 TAAAGAGCTTGACCGTATTCAAAAAGCCGTTGCAATGGCACAAAAAAATCATCATTATT  
 GATTAATGCAGGTCATGGTTTGACGCGTGATAATGTTGCAGCGATTGCCCAAATTGATGG  
 TATTCATGAGCTGAATATCGGGCATGCATTGATTTAGATGCGATATTTATGGGGCTTGA  
 TAATGCAGTCAAGGCAATGAAAATGGCTTTTATTCAAGATAAAACGACCAATCATTTGATG  
 CGTTAGAAAGAAAAATCGTAAATAATGATGACTATTGTGTAATATTATGTTTTTTGTTCA  
 60 AAAAAAGGTTGTAAAAAAATTCAATTACCATTAAAGCTAAGCCCAAGCCACAATGAATA  
 CCTATTGGTTTGACTCATTAGTCACTAAGAATCTGCAAAATTTGTAAACAGATTATTGGC  
 AGGTCTTGGATCGCTATGCTAAAAATAGGTGCGGTAATCTTGAAAAACCAACCATTCCTTG  
 GAGGAATTTATGAAAAAGGATATAAACGCTCTTGCGGTCATCGCAGCCGTTGCAGCTCC  
 AGTTGCAGCTCCAGTTGCTGCTCAAGCTGGTGTGACAGTC

65

SEQ. ID NO:40

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the copB gene  
 from *Moraxella catarrhalis***

70 GATGCTGTAAAGTGGGTATTGGTCTGTTCTATTTGTACAACCCGATTGTTGCAGGC  
 ATTGGCGTCCCGCAGATAAGTGCCATTGATAGTGTGGCAAGTGCGTTAAAAGATCGCATT  
 CCTTTGATTGCCGATGGCGGTATTCGTTTTTCGGGTGATATCGCCAAAGCCATCGCAGCA  
 GCGCTTCATGTATTATGGTGGGTAGCTTGTGGCAGGTACCGAAGAAGCACCTGGTGAG

GTGGAATTATTCCAAGGTCGTTATTATAAGGCTTATCGTGGTATGGGCAGCTTGGGGGCA  
 ATGTCTGGTCAAAATGGCTCATCGGATCGTTATTTTCAAGATGCCAAAGATGGTGTGAA  
 AAACGGTTCAGAGGGTATCGAAGGCCGTGTTCTTATAAAGGCCCTGTGGCAGGCATC  
 ATCGGTCAATTGGCAGGTGGTCTAAGATCATCCATGGGTATACAGGTTGCCAGACCATC  
 5 GAACAGATGCGTAAGAATACCAGCTTTGTCAAAGTGACTTCCGCAGGCATGAAGGAATCG  
 CATGTACACGATGTACAGATTACCAAGAAGCACCCTTATCGCCAAAATTAACCTCTAT  
 TAATAGCAAATACAAGCACTCATTAGATAGGGTGGGTGCTTTTTAGAGCATAAAAAATAA  
 ACTGACACATGACTTATTGTCATATTTTAAATGCTTTAATTTAGATTTTAAATTTAG  
 10 ATAATGGCTAAAAATAACAGAATATTAATTTAAAGTTTCAAATCAAGCGATTAGATGA  
 AATTATGAAAATAAATAACAATAATTCTGATTTATTTAACCAATAATATCAATTATCAT  
 TTACAAGAAAAATTTTTTTTGATAAAATTCTTACTTGTACCTTGCTATTTTTTCTTATTT  
 ATCATTTTTTGGCGGTATTTTCGTTGATTTTAGTAAGTAGATGAGCAAGGGATAATTTGAC  
 AAAACAAATTTGATTTCAAGCCTCATAATCGGAGTTATT

15 SEQ. ID NO:41

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the D15 gene  
 from *Moraxella catarrhalis***

AAAACGGTGATGTCTTCACTGCTATTTCATGGTGAACCAATCAATGATTGGCTAAGTGCC  
 ACCAAGATTATTCAGGCAAATCCAGAAACCATGCTTGATGTGACAGTCATGCGTCAAGGT  
 20 AAGCAGGTTGATTTAAAATTAATGCCCCGTGGTGTAAAGACACAAAACGGCGTAGTCGGT  
 CAACTGGGTATTGCCCCCAGATTGATATCGATACGCTCATTCTGATGAATATCGTATG  
 ACGATTCAATATGATGTCGGTGAGGCATTTACTCAAGCCATCCGACGAACTTATGATTTA  
 TCAATAATGACCTTAGATGCGATGGGTAAAGATGATTACAGGATTGATTGGCATTGAAAAT  
 CTATCAGGTCCCATTTGCCATTGCCGATGTTTCTAAGACCAGTTTTGAGTTGGGATTTCAA  
 25 GAAGTGTTATCGACAGCCGAATCATCAGTTTAAAGCTTGGCAGTACTGAATCTTTTACCC  
 ATTCAGTGTTAGATGGCGGGCATTGGTATTTTATACCTTATGAATGGATTATGGGCAAA  
 TCTATGAATGAAGCGGTGCAGATGGCAGCATTTAAAGCGGGTGCCTTATTTCTTTTTGT  
 TTCATGTTACTTGCAATCAGTAACGATATCATGCGATTTTTTGGCTAAGTTCTGATTTAT  
 CGTACCATTAAACAAAATTTTTGGCTTTTTTAAAGCTGAAATACTTGCCAAATTTAACTTTT  
 30 TGGCTTACCTTTACACAATATAAATTTGGGTGTAGAAAATTTTGGATACATTTTTTATACC  
 TTATTTTTTAGAAAATTTTAAAAATTAAGTTTGGATAGACTTATGCGTAATTCATATTTTAA  
 AGGTTTTTCAGGTCAGTGCAATGACAATGGCTGTCATGATGGTAATGTCAACTCATGCACA  
 AGCGGCGGATTTTATGGCAAATGACATTGCCATCACAGGACTACAGCGAGTGACCATTGA  
 AAGCTTACAAAGCGTGCTGCCGTTTCGCTTGGGTCAAGTG

35 SEQ. ID NO:42

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the omplA gene  
 from *Moraxella catarrhalis***

ACTTGGCGAAAATACCATTTATATCGATTGTGATGTTATACAGGCAGATGGCGGTACACG  
 40 CACAGCCAGTATCAGTGGTGCTGCCGTGGCACCTATTGATGCTTTAGAACCTTGCAGCG  
 TCGTAAAAAGCTTACCCAAGATCCGCTTTTGGGCTTGGTGGCAGCGGTTTCTGTGGGTGT  
 TAATCAAGGCCGTGTATTGCTTGATTGGATTATGCTGAAGATTCAACTTGTGATACCGA  
 TTTAAATGTGGTCATGACGCAGGCAGTGGGTTTATTGAGATTCAAGGCACAGCAGAAGA  
 AAAGCCATTTACTCGTGCTGAAGCTAATGCGATGCTTGATTTGGCAGAGCTGGGAATTGG  
 45 GCAGATTATCGAAGCCAAAAGCAAGTATTAGGCTGGTATATGCTAATCGTTGAAGATA  
 ATGGCGTGATCATCACATTAAATGGACAAGTAAAGACCCATTATTTTGGTGGTTCGATGA  
 TATTGCTGCTGCTGGGTGTCTTGGTGGCAATCATTTGTTTATTGACCCGTTTTTTATG  
 CAATCGGTGCGTTGGCTTTATTTGCAAGTTGTGGTATTTGTGTTAATATTCAAAGGCAAA  
 AAGCCAAAATTTGTCATATGTTTTCAAAAGGTCGCTTGAAGATTACGTCCAAACGCTTTG  
 50 AGATTCAATAACAAATCACTAACCTTATCAGCATCGGCAACAATATCTGCTAAAGATAACA  
 AAATGACAATTGTTGATCGGGGCATTGAATATCATTTTACAGGTTTGTGCTGATGACCGTG  
 AAATTAATATAGCCAAACAGGTACTTTTGGGAAAGTCAATCAAAACCAATGCGGTGGCGG  
 TAACATTGGCTAAGTAGTTGTTGTGATACAGACAGGTTGGATGGTCTTTAACTCCACCCA  
 CCTAACTTTTTCTTTGTTTGGATTAAAGAGTATGTTATGATGGGCAGGATTTTATTTTAA  
 55 GTCATCATTTAATGCAATCAGTTGTCCAGAGTAGCCGTTT

SEQ. ID NO:43

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the hly3 gene  
 from *Moraxella catarrhalis***

GTGATCGGCAACACCCACCATTCAGGAGCAACCAAAATTGCCCGTGCCTTGCCTGTCTT  
 60 GGTGGTATCATTTGGCAGGGCAATGTGGCTAAGTAGTGGTGTGCCATCAGGTGCGGTGGT  
 GGTGAGTGATCGATTGCTTATTGTCATAAAATTATCCTTTTGGGTGGATGATATCAATG  
 AAATACCCCTACGGTTGTATGGAATTTTCCATTGTACCACGGTATTGGTCTTTTTTAAAT  
 TAACAAGCAGCTTCTAGCAAGTCAAAGTTTATGCCTATTTTTTCAGATTTTAAAGGTAC  
 65 AATAAAGCCAATTGTTAATAATATGGTATTGTGATGATTATGATGAATTGCGACCAAAA  
 TTTTGGGAAAATTATCCCTTAGATGCGTTAACAGATGCTGAATGGGAAGCATTATGTGAC  
 GGATGTGGCGCGTGTGTTTGGTGAAATTTCTTGATGATGACAATGTTAAATTGACCGAA  
 TATACCGATGTTGCCTGCCAGCTATTGGATTGCTCAACAGGATTTTGCCAAAACCTATGCC  
 AAGCGTCAAACGATTGTGCCAGATTGTATTGCTTAAACCTGATATGCTGCCTGATATG  
 70 CTGTGGTTGCCACGCCATTGTGCTTATAAGCGGTTGTATCTTGGGCAAAATCTGCCAGCA  
 TGGCACAGGCTCATTAAACATAGCCAAAACCATGGTGCAGGATTGCGAAAGTTTCAACT  
 GCTGGGCGATGTGTGAGTGAGCTTGGTATGAGTGATGAAGACATAGAAAGGCGAGTGGTG  
 AAATGGGTAAACCTTGACATGATTGTTGACATGATTGACAGACAATAAAAATTGGCAAA  
 TTTGATAAAATTGGTGTATGTGTGTGATTTTATCAAAAGCACTTGAATAAAACCGAGTGA

TACGCTAAATTGTAGCAAACCAATCAATTCATCATAATTTTAATGAACACGAGGTAAAT  
TATACTGTCTATGTCTGATGACAATTCAGCACTTGGTCTG

## SEQ. ID NO:44

5 **Nucleotide sequence of DNA region (1000 bp ) up-stream from the lbpA gene  
from *Moraxella catarrhalis***

TAACAAAGGCAACCCAACACGCAGTTATTTGTGCAAGGCGGTCAAGCGGATGTCAGTAC  
TCAGCTGCCAGTGCAGGTAAATTCACCTATAATGGTCTTTGGGCAGGCTACCTGACCCA  
GAAAAAGACAAAGGTTATAGCAAAGATGAGGATACCATCAAGCAAAAGGTCTTAAAGA  
10 TTATATATTGACCAAAGACTTTATCCACAAGATGACGATGACGATGACGATGACGATAG  
TTTGACCGCATCTGATGATTCACAAGATGATAATACACATGGCGATGATGATTTGATTGC  
ATCTGATGATTACAAGATGATGACGCAGATGGCGATGACGATTCAGATGATTTGGGTGA  
TGGTGCAGATGATGACGCCGAGGCAAAGTGTATCATGCAGGTAATATTCGCCCTGAATT  
TGAAAACAAATACTTGCCCATTAATGAGCCTACTCATGAAAAACCTTTGCCCTAGATGG  
15 TAAAAATAAGGCTAAGTTGATGTAACTTTGACACCAACAGCCTAACTGGTAAATTAAA  
CGATGAGAGAGGTGATATCGTCTTTGATATCAAAAATGGCAAAATTGATGGCACAGGATT  
TACCGCCAAAGCCGATGTGCCAAATCGTGAAGAAGTGGGTAAACAACCAAGGTGGCGG  
TTTCTTATACAACATCAAAGATATTGATGTTAAGGGGCAATTTTTTGGCACAAATGGCGA  
AGAGTTGGCAGGACGGTTACATCATGACAAAGGCGATGGCATCACTGACACCGCCGAAAA  
20 AGCAGGGGCTGTCTTTGGGGCTGTTAAAGATAAATAAAGCCCCCTCATCATCGTTTAGT  
CGCTTGACCGACAGTTGATGACGCCCTTGGAATGTCTTAAACAGCACCTTGAAACAGT  
GCCTTGGGCGAATTCTTGGATAAATGCACCAGATTTGCCTCGGGCTAATATCTTGATAAA  
ACATCGCCATAAAATAGAAAATAAAGTTTAGGATTTTTTTT

## 25 SEQ. ID NO:45

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the lbpB gene  
from *Moraxella catarrhalis***

CAGCTTGTACCATTTGGTGAATATATACCATTTGGTGGTTTGTGGATATTTTACCAGGG  
CTTGAGGGTGTGCGTAGCCTAAGCCGTGGCGATGATAAGCAACCACCGCTCAAATTGGGC  
30 GCGGCGGTGGGCGATACGATTGGTGGCGCAATTTGTTATGAGGTGGCATATCCTGAGACG  
ACGCGTAAAAATGCACCTTGGCAGTAATTTTTTTTAAACCGTCTCAAACGATGCTTGGTTT  
GGTACAACAGCAGGTCCCTTTCAGCATTTACAAATGGTGCAAATGCGAAGCTTGGAGACG  
GGGCGATGGTTTGTGCGTGCAACAAACAACGGAGTGACTGCATTAATTGACCATCAAGGA  
CGGATTATCAAGCAGATACCGCAGTTTCAGCGAGATATTTTGCGAGGTGATGTACCCAGT  
35 TATGTTGGACACACGCCCTTATATGGTTTGGGGGCATTATCCCATGTTGGGGTTTTCTTTG  
GTGCTGATTTTTCTTAGTATCATGGCAAAGAAAATGAAAAATACCACCGCCAAACGAGAA  
AAATTTTATACCGCTGATGGTGTGGTAGACCGCTGAATTGTGCCACTTTGGGCGTTAGAG  
CATGAGCAAGATTAGGCGTTGGGTGAGCTTTGGTTGTATTACTCATCAGCCTACCCGAAA  
CCTGCCAAACATCACCGCCCAAAACCTAAACATACAATGGCTAAAAATATCAGAAAATAA  
40 CTTGCTGTATTGTAAATCTTATGTTATCATGTGATAATAATTATCATTAGTACCAAGAT  
ATCCATTACTAACTTCATCCCCATCTTAACAGTTACCAAGCGGTGAGCGGATTATCCG  
ATTGACAGCAAGCTTAGCATGATGGCATCGGCTGATTGTCTTTTGCCTTGTGTGTGTT  
TGTGGGAGTTGATTGTACTTACCTTAGTGGTGGATGCTTGGGCTGATTTAATTAAATTTG  
ATCAAAGCGGTCTTCACAACACACCAACGAGATATCACC

45

## SEQ. ID NO:46

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the tbpB gene  
from *Moraxella catarrhalis***

AGTTTGCCCTGATTTTGAGAGCCACTGCCATCATGAATTTGTTGGCGTAAACACCACTCG  
50 TATTCTTCTTCGGTTTCCCTTTCCATGCAAACACAGGGATACCAGCGGCCCGCCATGGCA  
GCGGCGGCGTGGTCTTGGGTGCTAAAAATATTGCATGATGTCCAGCGAAGTTCTGCACCC  
AAGGCAACCAAAGTCTCAATCAGCACCGCTGTTTGAATGGTCATGTGGATACAGCCTAGG  
ATTTTAGCACCCCTTAAGTGGTTGCTGGTCTTGATAGCGTTTCTTAACCCCATCAGGGCT  
GGCATCTCAGCTTCTGCCAAGGCAATCTCACGGCGACCATAATCGGCTAAACGGATATCA  
55 GCGACTTTATAATCGGTGAAGTTTGGGTGGTACTTGGATTGATTGAGGTAGGCATATCT  
TTATTCTTAAGCTATTTTAAAGTATTTTAAACAATAATTTTGATGAATTTGAGATAATTG  
ATGCTAAAAGGTTGAATGACCAACCATCGCTAACAATCAAGAAAAGACATTTTAAGCAT  
AAAAAGCAAATGTGTCTTGATGGCTTATTATAACAGTTATTATGATAAATTTGGGTAGAA  
AGTTAAATGGATCGTTGGGTAAGTTTGGTGGCTATCCTTAATTAATTATAATTTTTTAAT  
60 AATGCTTTTACTTTATTTTAAAAATAGAGTAAAAAATGGTTGGCTTTGGGTTTTTATCTC  
ACTATGGTAGATAAAATTGATACAAAATGGTTTGTATTATCACTTGTATTGTATTATAA  
TTTTACTTATTTTACAACTATACACTAAAATCAAAAATTAATCACTTTGGTTGGGTGG  
TTTTAGCAAGCAAATGGTTATTTTGGTAAACAATTAAGTTCTTAAAAACGATACACGCTC  
ATAAACAGATGGTTTTTGGCATCTGCAATTTGATGCCTGCCTTGTGATTGGTTGGGGTGT  
65 ATCGGTGTATCAAAGTGCAAAAGCCAACAGGTGGTCATTG

## SEQ. ID NO:47

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the tbpA gene  
from *Moraxella catarrhalis***

70 TTGGGGGCGGATAAAAAGTGGTCTTTGCCCAAAGGGGCATATGTGGGAGCGAACACCCAA



ATCTATGGCAAACATCATCAAAATCACAAAAATACAACGACCATTGGGGCAGACTGGGG  
 GCAAATTTGGGCTTTGCTGATGCCAAAAAGACCTTAGCATTGAGACCTATGGTGAAAAA  
 AGATTTTATGGGCATGAGCGTTATACCGACACCATCGGCATACGCATGTCGGTTGATTAT  
 AGAATCAACCCAAAATTTCAAAGCCTAAACGCCATAGACATATCACGCCTAACCAACCAT  
 5 CGGACGCCCGAGGGCTGACAGTAATAACACTTTATACAGCACATCATTGATTTATTACCCA  
 AATGCCACACGCTATTATCTTTTGGGGCAGACTTTTATGATGAAAAAGTGCCACAAGAC  
 CCATCTGACAGCTATGAGCGTCGTGGCATAACGCACAGCGTGGGGGCAAGAATGGGCGGGT  
 GGTCTTTCAAGCCGTGCCCCAATCAGCATCAACAAACGCCATTACCAAGGGGCAAACCTA  
 10 ACCAGTGGCGGACAAATTCGCCATGATAAACAGATGCAAGCGTCTTTATCGCTTTGGCAC  
 AGAGACATTCAAAATGGGGCATCACGCCACGGCTGACCATCAGTACAAACATCAATAAA  
 AGCAATGACATCAAGGCAAATTATCACAAAAATCAAATGTTTGTGAGTTTAGTCGCATT  
 TTTTGATGGGATAAGCACGCCCTACTTTTGTGTTTGTAAAAAATGTGCCATCATAGACA  
 ATATCAAGAAAAAATCAAGAAAAAAGATTACAAATTTAATGATAATTGTTATTGTTTAT  
 GTTATTATTTATCAATGTAAATTTGCCGTATTTTGTCCATCACAAACGCATTTATCATCA  
 15 ATGCCCAGACAAATACGCCAAATGCACATTGTCAACATGCCAAATAGGCATTAACAGAC  
 TTTTATTAGATAATACCATCAACCCATCAGAGGATTATTTT

SEQ. ID NO:48

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the ompE gene  
from *Moraxella catarrhalis***

20 AAAGACATTACACATCATCATTCAAACGCCCAACCATGTACCTCTGCCCCGTGGTCGCAC  
 GCCAACGCTTTTTGATGCGGTGCGTTGGGTTTACAGATGGCTTGTCAATCATTTGGTTTTAT  
 TAAAAATTCATACCTTTGGTAGTTTGGCTTTACCTGATATGTCATTTGATTATCGAAACAA  
 TACGCAGTTGACCAAACATCAATTTTAGCCATTGCCAAGCACTCAATATTACCGCTCA  
 25 TACGACCATGCTTGGTATTAAATCATCACATAAAGATACTTTACATCCATTGAAATTGAC  
 ATTACCCAAATACGGCCATGCCTCAAATTATGATGATGAATTGGTGCAAAACAATCCATT  
 GGCTTATTTTATCAACTGTCTGCCGTCTGCCGATATTTTATACCCAAACGGTTTGTAT  
 TGTTGGCGGTGAAAGCTCAGGGAAACTACCTTGGTGCAAAACTTGCCAATTATTATGG  
 TGCCAGCATCGCACCTGAAATGGGTGCGATTATACACACACTCCCATCTCGGCGGTAGCGA  
 30 ACTTGCCCTTCAATACAGCGACTACGCATCCATTGCCATCAATCACGCCAACGCTATCGA  
 AACCGCTCGTACCACTGCCAGCTCTGCTGTTACACTGATTGATACTGATTTTGCAGCAAC  
 GCAAGCATTTTGTGAAATTTATGAAGGGCGAACGCATCCGCTTGTGCGAGAAATTTGCTAA  
 ACAAAATGCGATTGGATTTTACGATTTATTTAGATAATAATGTTGCTTGGGTCGCTGATGG  
 CATGCGTAGGCTTGGTATGATATTAACGCAGTTTGTTCGCCAATAAATGCTTGAGAT  
 35 TTTGGCAGATATGATATTAGTTATCATATCATTAATGACACCGACTACCACAAACGCTA  
 TCTACAAGCATTAAGCTTGATAGACAATCATATTTTAAATCATTTTACAAAAATTCATGA  
 CAATTAATTAGGGAAATCTGATGAAATTTGATATTTTAG

SEQ. ID NO:49

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the uspa1 gene  
from *Moraxella catarrhalis***

GGATGTGGCATATCTGCCCATCGACCCAATACACATCGGTGCGAGGCTATCAAGATGTGGT  
 ACGAATTAATAGCCAGTCAGGTAAGGGCGGTGCTGCGTATATCTTGACGCGGCATTTTGG  
 45 TTTTAATTTACCACGCTGGACACAGATTGATTTTGTCTGTTGGTACAGGCTTATGCAGA  
 AAGTATGGCGCGTGAACATAAACTGATGAGCTGCTTGAAATTTTACCCAAGCGTATCT  
 TAAGCAAGATAAATTCGCGCTAAGTGACTATACCATCAGCAATAAAGCGGATGCTGTCAG  
 CTTCCAAGGCCAAGTAGCGACACCCAAAGCGGTGTTGAGGTGATTGGTCAAGGCAATGG  
 TGCGTTATCTGCGTTTATTGATGGCTTGGTGAAATCCACAGGCAGACAGATTCATGTCAC  
 CAATTACGCCGAACACGCCATCGATAACAAAACCCATCAAAAACCGATACGGATAACCA  
 50 AACCGATGCCGCGGTGCCGCTTATATCCAGCTGTCGGTAGAGGGGCAGATTTATTCAGGC  
 ATCGCCACTTGCCATAGCACCGTATCCGCCATGCTAAAAGGTGCATTATCCGCTTTGGCA  
 CAGGCGTGGTAATCTGACCCAATCAAAATCCTGCATGATGGCAGGATTTTATTATTTAGT  
 GGGCTGCCCAACAATGATGATCATCAGCATGTGAGCAAATGACTGGCGTAAATGACTGAT  
 GAGTGTCTTATTTAATGAAAGATATCAATATAAAAGTTGACTATAGCGATGCAATACAG  
 55 TAAAAATTTGTTACGGCTAAACATAACGCGGTCCAAGATGGCGGATATCGCCATTTACCA  
 ACCTGATAATCAGTTTGATAGCCATTAGCGATGGCATCAAGTTGTGTTGTTGATTGTCA  
 TATAAACGGTAAATTTGGTTTGGTGGATGCCCCATCTGATTTACCGTCCCCCTAATAAGT  
 GAGGGGGGGGAGACCCAGTCATTTATTAGGAGACTAAG

60 SEQ. ID NO:50

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the uspa2 gene  
from *Moraxella catarrhalis***

CCCCAGCTTTCCGTTTGTGTGCTGCTGGTGTGCGGGCGGTATACCATGCTGGTGGTGGT  
 CCGATGGTGTGGGCGTGTATCAGATATTGGGCGAGTCTATCGATGATGCGGTGGGTGAAT  
 65 GCTTTGATAAAAACGGCAAAAATGCTCAAACTGCCCTATCCTGGTGGCCCCAAATATCGAAA  
 AATTAGCCAAAAACGGCAACCCACACGCCTATGAGCTGCCAAGACCCATGCAGCATAAAG  
 GGCTGGATTTTTCGTTTCAAGTGGCATGAAAACCGCCATTCTAATCTCATCAAGACACAC  
 CAAACGCCCAAGCGACCCCGCCACACGAGCAGACATCGCCGCAAGCTTTGAGTATGCGG  
 TGGTGGATACTTTGGTCAAAAAATGCACCAAGCACTACAGATGACAGGCATTCGCCAGC  
 70 TGGTGGTTCGAGGGGGCGTCTCTGCCAATCAGATGCTACGCCGACCCCTGACCGAGACGC  
 TCCGCCAAATCGATGCGTGGTGTACTATGCCCCGACCGAGCTATGCACGGATAATGGTG  
 CGATGATCGCCTATGCTGGCTTTTGTGCGCTCAGCTGTGGACAGTCGGATGACTTGGCGG  
 TTCGCTGTATTCCCCGATGGGATATGACGACGCTTGGCGTATCGGCTCATAGATAGCCAC

5 ATCAATCATACCAACCAAATCGTACAAACGGTTGATACATGCCAAAAATACCATATTGAA  
AGTAGGGTTTGGGTATTATTTATGTAACCTATATCTAATTTGGTGTGATACTTTGATAA  
AGCCTTGCTATACTGTAACCTAAATGGATATGATAGAGATTTTCCATTTATGCCAGCAA  
AAGAGATAGATAGATAGATAGATAGATAAGAACTCTGTCTTTTATCTGTCCGCTGATGCTT  
TCTGCCTGCCACCGATGATATCATTATCTGCTTTTAGGCATCAGTTATTTACCGTGA  
TGACTGATGTGATGACTTAACCACCAAAGAGAGTGCTAA

SEQ. ID NO:51

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the omp21 gene  
from *Moraxella catarrhalis***

10 GAGTGAACCTTTATTGTAAAAATATGATTTCATTAAAGTATCAAAATCATCAAACGCAGCATC  
AGGGTTTGCTAAATCAATTTTTCACCATAATTATAGCCATAACGCACAGCAAGCGTAGT  
TATGCCAGCGGCTTGGCCTGATAAAATATCATTTTGGAAATCACCACCATAATGGCATC  
15 AGTCGGTGGCAGTGCCAGTGATTGACACAGGTATAATAAAGGCGTTGGGTGCGGGCTTTT  
GACGCTGAGCGTATCACCGCCAATCACTTGGTCAAACAGTGTGAGCCATCCAAAATGTGA  
TAAAATTTTAGGCAAATAACGCTCAGGCTTATTGGTACAAATTGCCAAATAAAACCCCGC  
TGCTTTTAATCGTTCAAGCCCTTGTATAACCCCTGCATAGCTTTGCGTATTTTCAATTGT  
TTTATGGGCATATTCTGCCAAAATAACTCATGGGCATGGTGAATCATAGTCGTATCATA  
20 GATATGATGTGCTTGCTCGCTCAACCAATTTTAGCGAACCATTGCCACCCAGCT  
TTTGATGATATCAATTGGCATAGGCGGTAAGTTAAGCTTGGCATACATGCCATTGACCGC  
CGCCGCCAAATCAGGGGCACTATCGATAAGCGTACCATCCAAATCAAATATAATCAGTTT  
TTTGCCAGTCATTGACAGTGTTCATGCTTTTCCCTTATTCTTAAAATTGGCGGCTGTT  
TGGTATTTTAAATCAGTCAATTTTACCATTGTGCATATAATGACAAAGTACAAATTT  
25 AGCAATATTTTAGTGCAATTTTGGCGAAGTTTATGAAAAGTGGTCAATGGTTGCAAAA  
CTTTACACAGTACCTATAAACTTGCACAGTTAATAAGAAATATTTGTTACTATAGGGG  
CGTCATTTGGAACAAGACAGTTATTTGTAAATAGTTATTTGCAAAAGACGGCTAAAAGAC  
AGAACAGCGTTTGTTCAGTGATTAAGTAGGAGAAAAACA

SEQ. ID NO:52

30 **Nucleotide sequence of DNA region (1000 bp ) up-stream from the omp106 gene  
from *Moraxella catarrhalis***

TTGATCGGTTTTGCCCCACTGTTTCATGATTTACTCAAAACAGGCGGCTTGATCGTGCTG  
GCAGGTCTGACCCAAAACCAACCCAAAGCGGTCATCGATGCCTACTCGCCTTATGTTACG  
CTTGATACGCCATTTTGTATGCAGATGCCAAGACTGCCATTGGCAACGCCAAGCGGC  
35 ATCAAACCTACCAACCCATAAGCGATATGCCATGAGCCACAAACCTAAGCCAAACCCGCT  
ATATCAACAAGTTGAGCAGACCGCCAAGCGTTATTTTGGACATTTGGGCGATGCTCATAC  
TCATGATGTCTATGCCACTTTTTTGGCCGAATTTGAAAAACCGCTGCTCATCGCCGCACT  
CAATCACACGCACGGCAATCAGTCAAAAACCGCCCAAATCCTTGGTATCAATCGTGGCAC  
ATTACGCACCAAAATGAAAACCCATCACTTACTTTAGACCGCCAGTTATCGCCATGGATA  
40 TGGGCAGGTGTGCTCGCCTGCCGTATGATGGCGATGACACCCCATTTGCCCCATATCTGC  
ACGATTTGACATGATTTAACATGTGATATGATTTAACATGTGACATGATTTAACATTGTT  
TAATACTGTTGCCATCATTAACATAATTTAGTAACGCATTTGTAAAAATCATTGCCCCCT  
TTTTTTATGTGTATCATATGAATAGAATATTATGATTGTATCTGATTATTGTATCAGAAT  
GGTGATGCCTACGAGTTGATTTGGGTAAATCACTCTATTATTTGATATGTTTTGAAACTA  
45 ATCTATTGACTTAAATCACCATATGGTTATAATTTAGCATAATGGTAGGCTTTTTGTAA  
AATCACATCGCAATATTGTTCTACTGTTACCACCATGCTTGAATGACGATCCAAATCACC  
AGATTCATTCAAGTGATGTGTTGTATACGCACCATTTACCCTAATTATTTCAATCAAAT  
GCCTATGTCAGCATGTATCATTTTTTTAAGGTAAACCACC

50 SEQ. ID NO:53

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the HtrB gene  
from *Moraxella catarrhalis***

ACTATTCTGCTTTTTGTTTTTACGGAATGCGAATGCCAACTCACGCAACTGGCGATTAT  
CAACTTCAGCAGGTGCTTCGGTCAATGGGCAATCTGCCGTCTTGGTTTTTGGGAAGGCGA  
55 TCACATCACGGATTGAGCTGGCACCAACCATCAGCATAATCAGGCGATCTAGACCAAATG  
CCAAACCACCGTGCGGCGGTGCACCAAAACGCAATGCATCCATCAAAAACCTAAACTTAA  
GCTCTGCTTCTTCTTTAGAAATACCCAAGGCATCAAAATACCGCCTCTTGATGTCAACCG  
TATTAATACGCAGCGAACCAGCCACCAATTTCTGTGCCATTTAGTACCATGTATAGGCAA  
TGGATAGGGCGGTTTCGGGACTTTGTTTGAAGTTCCTCAACCGAGCCTTTTGGGCGTGTA  
60 AAGGATGATGAACTGATGTCCACTTACCATCATCAGTTTCCTCAAACATTGGAAAATCAA  
CGACCCAAAGCGGTGCCCATTCACAGGTAAATAAATTTAAATCAGTACCGATTTTAACAC  
GCAATGCACCCATAGCATCATTTGACGATTTTGGCTTTATCGGCACCAAGAAAATGATAT  
CGCCAGTTTGGGCATCGGTACGCTCAATCAGCTCAATCAAACCTCATCGGTCATATTTT  
TAATGATGGGTGATTGTAATCCTGATTCTTTTCAACGCCATTATTGATATTGCTTGCGT  
65 CATTGACCTTAATATATGCCAATGACGAGCGCCATAAATACCAACAAATTTGGTGTACT  
CATCAATCTGCTTGCGACTCATGTTACCGCCATTGGAATGCGTAAGGCAACAACACGGC  
CTTTAGGATCTTGGCGGGGCCCTGAAAATACTTTAAATTCAACATGTTGCATGATGTCAG  
CAACATCAATAAGTTTTAAGGGAATGCGTAAATCAGGCTTATCTGAGGCATAATCACGCA  
70 TGGCATCTGCGTAAGTCATGCGGGGGAAGGTATCAAACCTCA

SEQ. ID NO:54

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the MsbB gene  
from *Moraxella catarrhalis***

5 TGGATCATATTCTTTATTAATGGTACTGTTTAAACCTGTATTTTAAAGTTTATTGGGTCA  
TATTTTCAAGCTCATCCCATCGCTCAAGCTTCATCATCAAAAGCTCATCAATCTCTACCA  
ATCGCTCACCAGCCTTCGTTGCTGCCGCCAAATCGGTATTAACCATGAACCATCTTCAA  
TCTTTTTGGCAAGCTGTGCCTGCTCTTGTTCAGTGCAGCAATTTATTAGGCAAATCTT  
CAAGTTCACGCTGCTCTTTATAGCTGAGTTTGCCTTTTGGGCAACGCTGATTGAGGTG  
GTTTGATTTGGATGGGTTTACGCGGTTTTGTGCGCTTAGGTTTATTGTCTGTGGCGTGAT  
10 GAGCAAGCCATCTTTTCATGCTGTTGTACATAGTCTTCATAACCGCCAACATATTCCAAAA  
CGATACCGTCGCCGTACTTATCAGTATCAAATACCCAGTTTGGGTAACAACATTATCCA  
TAAAAGCACGGTCATGGCTGATGAGTAATACCGTGCTTTAAAATTGACCACAAAATCTT  
CTAAAAGCTCAAGTGTGGCATATCCAAATCATTGGTAGGCTCATCAAGCACCACAAACAT  
TGGCAGGTTTTAGCAATAATTGGCCATAAAACGCGTGCTTTTTTACCGCCTGATAGTG  
15 TTTTAACAGGTGTGCGAGCAGATTGGCGTGAATAAAAAATCTTGCAAAATAGCTTAAAA  
TGTGCGTAGTTTTTCCACCAACATCGACATGGTCAGAGCCTTCTGAAACATTATCTGCGA  
TAGATTTTTTCAAGGTCTAGGTCGTCTTTGAGTTGGTCAAAAAAGCAATATTTAGATTGG  
TGCCAAGCTTAACTGAACCTGACTGAATCGCTGAATCATCCAAACCCAAAATGCTTTTAA  
TTAAGGTTGTTTTACCAACGCCATTTTTGCCAATGATACCAACTTTATCACCACGAACAA  
20 GCAGCGTTGAAAAATCCTTAAGGTTTTATTGTCGTAT

SEQ. ID NO:55

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the PilQ gene  
from *Moraxella catarrhalis***

25 CAACTTGAAAATCAGCTCAATGCTCTGCCACGCACAGCACCGATGAGCGAGATTATCGGA  
ATGATAAATACCAAAGCACAAGCGGTTAATGTGCAGGTGGTGAGTGCATCAGTTCAAGCA  
GGTCGTGAACAGGATTATTATACCGAACGCCCTATCGCAGTGAGTGCGACAGGGGATTAT  
CATGCTTTGGGTGCGATGGTTACTTGAGTTGTCAGAGGCTAACCATTTGCTGACAGTGCAT  
GATTTTGATCTGAAGGCTGGTTTGAACCATCAGCTGATGATGATTGTTTCAATGAACT  
TATCAAGCGAACAAACGCCCAAACAGTTGCTCAGCAGGTGCCTGATGTTCAATGAATA  
30 TTATCGGTGGGGCATTTTGGGTGCTTGGATTGGGTGGGATTGGATGTGCTGATAGCAC  
CAGTCAAGTTGTTGATGATAAGCTTGACATATTACCCATGAAGAGCGTATGGCGATCAG  
TGAGCCTGTGCCGATACCCCTTATCTGTGCCGATGATATATCAGCAAGGCAAAGATCCTTT  
TATCAATCCTTATAGAAATGTTGAGGTTCTTGATACCAATCATGCCGCTGATCAGCAAGA  
TGAGCCAAAACCGAATCTACCAAAGCTTGGCCTATGGCAGACACTATGCCATCTCAGCC  
35 ATCTGATACTCATCAGTCTGCCAAGGCTCAGGCACAAGTCTTCAAAGGCGATCCGATAGT  
CATTGATACCAACCGTGTTCGAGAGCCTTTAGAAAGCTATGAGTTATCAAGCCTACGCTA  
TCATGGTCGTATTTTTGATGATGTTAGACTTGTGGCACTCATTATGAGTCCTGATGGCAT  
CGTTCATCGTGTGAGTACTGGACAATATCTTGGTAAAAATCACGGAAAAATTACCCATAT  
TGACAGTCGTACGATACATCTGATTGAAGCGGTCGCTGATACACAAGGTGGCTATTATCG  
40 CCGTGATGTAAACATTCAATTTTATTTCATAAGCAATGACAC

SEQ. ID NO:56

**Nucleotide sequence of DNA region (1000 bp) up-stream from the lipo18 gene  
from *Moraxella catarrhalis***

45 TTCATGCAACAAGCGACCATCTTGGCCGATGATACCATCCTGCTCACCTAAGAAAAATCAG  
TTTATCAGCTTGCAAGGGCAATGGCTGTGGTCAGTGCTACATCTTCTGCCAATAGATTAAA  
AATTTCCGCCCGTAACCGAAAAACCTGTCGGTCCCTAGTAGGACAATATGGTCATTATCCAA  
ATTATGGCGAATGGCATCGACATCAATTGAGCGTACCTCACCTGTCATCTGATAATCCAT  
50 ACCATCTCTGATGCCGTAAGGGCGAGCGGTGACAAAATTACCCGAAATGGCATCAATACG  
AGATCCGTACATTGGGGAGTTAGCAAGCCCCATCGACAGCCGAGCTTCGATTTGTAGACG  
AATTGAGCCGACTGCCTCCAAGATGGCAGGCATAGATTTCATACGGTGTACACGCACATT  
CTCATGTAGGTTTGATATCAGCTTGCGATTTTGTAAATTTTTTCCACTTGTGGGCGTAC  
ACCATGCACAAGCACCAATTTGATGCCCAAGCTGTGTAGCAGTGCAAAATCATGAATCAG  
55 CGTACTAAAATTGTCACGAGCGACCGCCTCATACCAAACATAACCACAAAGGTTTGGCC  
ACGATGGGTGTTAATGTACGGGGCAGAATTACGAAACCAATGCACAGGTGTGAGTGCAGG  
AGTGTCTGATAGGTGCTGACAGAATTTCATGAATGCTCCAAGAGTCAATGGCTGGTAAA  
ATAAGAATGGCGAACAATATATGGCGAGAGCGTCTGATGTTGGTCAAATGTCCCATTAAAT  
AACTATCAAGATACCATCATACCATAGCAAGTTTTGGGCAGATGCCAAGCGAATTTATC  
AGCTTGATAAGGTGGCATATGATAAAATCTACCATCATCGTCGCCAGTTTTGAGCATGT  
60 GTAAGTAGTTACCATAATTAAACAGTCAAGAAATTCACACCGTCAATCAGCTGTGCTATG  
CTTATGGGCACATAAACTTGACCAACACAGGATAAATTTA

SEQ. ID NO:57

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the lipo11 gene  
from *Moraxella catarrhalis***

65 GGCATACTTTTGCCATGCTTTATTTTGGCATAACTGCTATAAGCCCATTTGCTACTTTTTTA  
TCATTTATCCATATGTCCAATAATGTGCTTTATGTAATTTAGGCACACTATTAACCTCGTG  
CCACTGTTAACATTCAGCATAAAAAATCTTAACAATGAATCAAAGCATCGTATTGGCTGTT  
AAATGATAAGCTTATATTTATTTAAATTCAGACTAAATGATTGTAATATGGACATATCAA  
70 GGTTGAAATCAAAAATTTTGGAGAGTTATGTACGATAATGATAAAAAATTGACCACCATC  
GTAGGGGTGTTGTATACGGTGTCTTATATTGCCATATGGTTGGTCAGTGGCTATATTTTA  
TGGGGCTGGATTGGTGTGACAGGATTTACTCGTGCGATACTTTGGCTGATCGCTTGGATG  
ATTGTGGGTACGATTGCTGATAGAATTCTGATACCGATTATTTTGACCGTCGTGGTTGGG  
TTATTTTCTATCTTTTTTGAAGGCGATAATTTGGTTATTTTTTTCACAAAAATCAT



GATTTTTTTTTGTAACTATCTAAAATATCAATTATGTTATATTATGTGATAAAAGATGGG  
CATGCTTAAGTTTTGGATTGCAAAAATCCTAATATCATCACTGACCAAAGCTGTGATGAT  
ATCAAAACTTTTATCAAAGTTCTTAGGGTATTATCAAGATATCATACCAAATGAATACTTA  
5 CCCAACTTACTATAAAAATCAAATGATATGACTGTGATTTTATTATCATAGATACAAAA  
TCAAAACGCATGAGCCAAAGGTATGATGAATGAATACAAAATTTTCGCACACATTATGACA  
ATCTAAATGTGCGCCAGAAACGCTGACATTGCGGTGATTTGGTGGGATAGGGGTCAAGCCA  
GTGCGATTAAGCTAAATTTTATGTGGGCAATCGCTGACTTTATTTTATTTGTGCCAGTT  
GGAACAATTCGTGGTCTAATGTATTTATTTAAGGAGATAA

10 SEQ. ID NO:58

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the lipo10 gene  
from *Moraxella catarrhalis***

15 TCTGGTCTACATCCCAAACCTATTTACACAAGAAACACTAAAGACAGTGGAGCAGATGACG  
CTCAAAAAGGCATCTTATAGTAATTTGACAGTTAATTTTCGTCAAGTGCTTGTACAAAAA  
TACACCATCGTGCAAGAAGTTTGTACCAATTTAAGCACAAATCATTTTGGCACACACTGTC  
AAGCAATGCTTCAGGCAAATTAGCTGCTGGTAAAGATACTTGGGTCAATCATGCAATCGCA  
TCAACCCTTCTTGCTGCGTTGAAGCGATAAGTTTGGCATCTTGCCAAAATTGACCATGGT  
TTAGACCCCTTGGCGTGGCTTGTGGTATCGCTCCACATGTCGTAGAGTAGATATTCGGTCA  
20 TATCAAAAGGGCGATGGAAATGTATGGAATGGTCAATACTAGCCATTTGTAGACCTTGTG  
TCATCAGGCTTAGCCCATGACTCATTAAACCTGTGCTGACCAAATAATAATCAGACACAA  
ACGCAAGTAGTGCTTGTATGAATGGCAACTGGCTGCTCCCAATATCAGCGATACGCACCC  
AATTGGCTTGGCGTGGACGCTCAGGCTTGGGTGTACAGGGTCTCGTGGTGTGACGGGGC  
GGATTTTCGACATGACGCTGACGCATAAATCTTGCTTTGAGTGGTTTCGGGAATTTTATGTA  
AATAATCCGCTTTGAGTTCTTGCTCGGTTTTTAGGCTTTCAGGGGGTGGATAATCAGGCA  
25 TGGTTTCTTGGTAATCAAGCCCGCCTTCCATGGGTGAAAATGAGGCAATCATCGAAAAA  
TGACCTGTTTCATTGGTTCGTATGATTACCGTTTTTGTGCGGTGGTTGGCACATATTGCACCG  
CAATGACTTCTCGAGCTGATAAACTGCGTCCATCACGTAAGCGGGCTACTTGATAGATGA  
CTGGTAGACGAATATCGCCACCTCGTAAAAATAACCATGTAGGCTATGACAAGGTTTAT  
CAATCGTTAATGTGTTAGCACCAGCAAGCAGCGCTTGGGCA

30

SEQ. ID NO:59

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the lipo2 gene  
from *Moraxella catarrhalis***

35 TAAATGACCTTACAAAATAAAATTATATGTTCAAAAATCGCTTAAGTATTGAAAAAGC  
TATAAAAACCTTATCTATTAAAGCATAAAAGATATTAAAGCATAAAAGACGAGAAAAGAGC  
AAGCGTCAATGATGATATTTATATAAAAACCTTATGAAATTTTCAATTTTATCGATT  
GATTCAGCTTGGCTATCGGTGGTCAACTTTGGCTGCCAAGACATCGCCGGCTTTTTGAAA  
AATCATCACAAATGGCAACAATGATGATGGTTGAAATCCACTTGACATATACCATGTTGCG  
40 ATGCTCACCATAGTTAATCGCAAGGCTTCCCAAGCCACCACCGCCAACCACACCTGCCAT  
TGCAGAATAACCAATCAAAGACACCAAGGTCAATGTGACCGCATTAATCAAAATGGGCAG  
GCTTTCAGCAAAATAGTATTTGCTGACAACCTGCCAATGCGTTGCACCCATAGATTGGC  
AGCTTCGGTCAGTCTGTGGGTACTTCTAATAAGCATTGGCACTCAAGCGTGCAAAAAA  
TGGAAATTGCTGCCACACTCAAAGGGCTGATGGCGGCTGTTGTGCCAAGGTTGTTCCAC  
45 CAAAAATCGTGTGACTGGCATGAGAATAATGAGCAAAATAATAAAAGGAACGGAGCGACC  
AATATTAATAATAACATCCAAAATTACAAATACACTGCGATTTTCAAGGATACGCCCTTT  
ATCGGTTAAAAATGCCAAAACCTATCGGTAGCCCAACCAAAACAGCGATGGCAGTGGC  
AGCAAGCCCATATAGATGGTTTCCCAAGTGGATTGGGCAACCATCTCCACATTCTTGG  
GTGCATTTCACTGACAAATTTTGTGACGATTTTCAATCCACATAGCCGATAATCTCAATAT  
50 TGACCCGATGGGTGGTTAAAAATTCTATTGCTTGATGACCGAGGTGCCTTCACCGATAA  
GCTCAGCAATGGTAAAGCCAAATTTTATATCACCTGCATAA

SEQ. ID NO:60

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the lipo7 gene  
from *Moraxella catarrhalis***

55 AGTAAACAATGGTAACAAATACAGCAGTGTCGCACAGTCCTCAGTACGATGATTCTGAAT  
TTGAATATGCAGGATTTTGGATACGATTTGTGGCATGTCTTGTGCGATAATTTAATTGTTA  
TGATTATAATTGCACCGTATTGGTTTTATAATTATCAGCAAATGATGGCCATGCCTGCTG  
ACCAAATACCGTTTATAGTGTTGGGGATGCCATCCTTTATAGTGCTGGGGATGCTATCC  
60 TAAACTTAGTGATGGCGGCGCGGTTGTTTTGGTTTGGGTAAAAAAGGTGCAACACCAG  
GTAAAATGCTCTTTGGGCTGCAAGTCCGTGATGCCAAAACAGGGCAATTTATCAGTGTGC  
CAAGGGCATTATTGCGATATTTTAGTTATCTGATTTTCATCCGTGATTCTTTGTTTGGGAC  
TTATTTGGGTTGGTTTTGATAAGAAAAACAAGGCTGGCATGATAAAATTGCCAAAACCTG  
TTGTGGTAAACGCATTTCGTGATGGGTGCGCAGTTAAACAATAAAACCATCAAACGCAA  
65 GCAGGGCGATGTGTTTGAAGCAGTTGGCGGTAGATAAGCTAAACAAGCAGGCTATGAAAT  
TATTTTAACCAACTTTACCACCCCATTTGTTGGTGAGATTGATATTATCGCCAGACAGCC  
TTTGGAGCAATCGCACCGTTTGGTGCAGCAAGATTTTGTACGGTATTTGTTGAAGTGGC  
TAGCCGAACAAGTTCTGTGTATGGTACAGCGCTTGAGAGTGTTACCTCAAAAAAGCAGGC  
AAAAATCTACCGAACAGCAGAACGATTTTAAATCAATTATCCCAAATATATTGATGATGC  
ATACCGTTTTGATGTCATGGTTTTGATTTGGTTGATGGATTGATTGAACATGAATGGAT  
70 AAAAAATGCGTTTTGATTGGCTCAATGGTCGTGAATTAAATCAATCAAGCAATCCGTAG  
CTTTACTATAAGATATATCCAGTAATATGGAAACATAGCA

SEQ. ID NO:61



**Nucleotide sequence of DNA region (1000 bp ) up-stream from the lip6 gene from *Moraxella catarrhalis***

CGTTTAGCTTCATACGCAGACCTTGTGCACCTTCGGGCAACCGAAGCATCACGCCAGCAT  
 5 CACGCATCCGCACAAAACCCATCATGCCATCAATTTGCTGCTGATATGATATACCCCCA  
 CCAAAGTAAACCGCTTAAATCGTGGAATAACGCCTGCTGCTGAGGGTGAGGCTTCAGGCA  
 AAACCAAGGTAACCTTATCCCCAACTTAAGTCCCATGTCAGAGACAATGGACTCACCTA  
 ATATAATACCAAACCTCGCCGATATGTAAATCATCCAAATTGCCTGCGGTCATATGCTCAT  
 CAATGATAGAAACTTGCTTTTCGTAATCAGGCTCAATGCCAGAAACCACGATTCCAGTCA  
 10 CCTGACCTTCAGCGGTAAACATACCTTGTAGTTGAATATAAGGGGCAACTGCTTGCACTT  
 CTGGATTTTGCATTTTGATTTTTTCGGCAAGTTCTTGCCAATTTGTCAAAAATTTCTGTTG  
 AGGTAAGTGAAGCTTGAGGCACCATGCCAAGAATGCGTGATTTAATTTACGGTCAAAGC  
 CATTTCATGACCGACAAAACCGTGATAAGCACTGCAACCCCAAGCGTAAGCCCAATGGTTG  
 AGATAAAAGAAATAAAGGAAATAAAGCCATTTTACGCTTAGCTTTGGTATATCTAAGCC  
 CAATAAATAACGCCAAGGGACGAAACATAAGCTGTGTTCCAAACGACCCCAACCGTGCTAG  
 15 TTAGCACTTTTTTGGACAAATACCAAACATCACATAACAAATGAATCATCAGGTTGGTT  
 TTGTTGCGCTTGTGTATCTGTATGATAAGTTTCTTGCTAAACAGCTTTTTTATGTGAGA  
 ATACAGAAAAGGTATATACTTATTTTAACTTTAAATAGATCTGCTTTTTTATACCGA  
 TGATTTGGCATGAAGTTTATCGGTCTGATGCTGGATATAAGTTTATCGGCTTGATATA  
 AATTTTAATTAATCATCAATTTTTTAAGGAATTTATCATTA

SEQ. ID NO:62

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the P6 gene from *Moraxella catarrhalis***

TAAGGATACCAGATTTTGGCTTGTCAATCGTTGTGTTAATCATTGTAACGGTTTATAGTG  
 25 ATTGTC AATTAATAAGGGTAAAAAAGTATTTATCAAGTAATAATCTTTCTTATATGTGAA  
 TATAATGACAAATTTATCACATTTTACAAGGATTTTATCAAGATTAGGATATGTTCC  
 AGCTTAATTATTAGTGATGAGCGTGTGATTATTTGGCATCGTTAAATTTATGAGTGCTAA  
 AATTGCCAATGATTAAAATTTTGCTAACATGATAGCCCCCTTTGGTAGGCTTTATTTGGT  
 ATTGATGAGCAATAATAATATACCGAGTTAAATGGATTAACTTAACATACGCCAAAACT  
 30 TAACAACGAAAAGTAGATGATTATGACAGATACAGTACAAAAGATACAGCACAGTCCCC  
 CAAAAAGTTTATCTAAAAGACTACACGCCGCCAGTATATGCAGTTAATAAAGTGGATTT  
 GGATATCCGCTTGTGTGATGATCATGCTGTGCTGGTGCCAACTTAAAATGACACGAGC  
 ACACGCAGGCGAGCTTCGGCTTCTTGGGCGAGATTTAAAGCTTAAAGCATTACCTAAA  
 TGGTCAGGAATTAGAGTTCGCGAGGCTATCATCTTGATAAGGAAGGCTTAACAATTTTGA  
 35 TGCACCAGATGTCGCGAGTGATTGAGACATTGGTTGAGATTTACCACAAAACCAACACAAC  
 ACTTGAAGGGCTATATCAAGCAGGAACAGGTGATGATAAGATGTTTGTGACACAATGCGA  
 ACCTGAGGGTTTTCGCAAAATCACCTTTTCCCTGACCGCCCTGATGTTTGTGACAGAATA  
 CACCACACGCCTAGAAGCACCAGCATTTTTAAACCTTGCTTGCCAATGGTAATTTGGT  
 TGAGTCAGGAGATGTGGATGAAAATCGCCATTATACCATTTGGCATGATCCTACCAAAA  
 40 ACCCAGCTATCTATTCGCCGCTGTCAATTGCCAATCTAGAAG

SEQ. ID NO:63

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the MsbB gene from *Haemophilus influenzae* (HiRd)**

AAATCAAGCGCCTGTGCCTGCTGGTGATGGTTGTGGAGACGAATTATATTCTTGGTTTGA  
 45 ACCGCCAAAACAGGCACTTCAGTGAGCAAACCTAAAGTTACACCGCCTGAGCCGTTTTT  
 GTGCCAACAGATTTTGAACCTACCGAATCGGAGAGAATGGTTAGAATAGCATTGAGGTAA  
 ATCAATATGGATATCGGCATTGATCTTTTAGCAATATTGTTTTGTGTTGGTTTTGTGCGA  
 TCATTTATCGATGCAATTGCTGGCGGTGGTGGATTAAATCACCATTCCAGCGTTACTCATG  
 50 ACAGGTATGCCACGCAATGGCGTTAGGCACCAACAAATTGCAAGCTATGGGCGGTGCA  
 TTATCCGCAAGCCTTTATTTCTTGGCAAAAAGAGCGGTCAATTTACCGGATATTTGGTTT  
 ATTTTGATTTGGGTTTTCTTAGGTTCTGCCCTAGGTACATTATTAATTCATCAATTGAC  
 GTGGCGATTTTCAAAAAAATGCTTCTTTTGTATTTAGCCATTGGTCTATATTTTTTA  
 TTTACTCCTAAATTAGGTGATGAAGATCGAAAACAACGATTAAGTTATCTGTTATTTGGT  
 55 CTTTTAGTTAGCCATTTTTTAGGTTTTATGATGGCTTCTTTGGGCCAGGGACTGGCTCA  
 ATCATGAGTTTAGCCTGTGTTACTTTGCTAGGATTTAATCTCCCGAAAGCGGCAGCACAT  
 GCAAAAGTGATGAACCTTCACTTCGAACCTTGCTTCTTTGCACTTTTCTTATTGGGCGGA  
 CAAATTCCTTTGGAAAGTGGGTTTCGTGATGATGGCTGGGAGCATTTTAGGTGCAATTTA  
 GGTGCCAAAATGGTGATGACGAAAGGTAAAACCTTGATTGACCGATGGTTGTTATCATG  
 60 TCTTTTATGATGACGGCTAAAATGGTTTACGATCAGGGTTGGTTTCATTTTAAATTCGGA  
 AAGCGCGCAAAAGTGCGGTAAATTAATTACATTTTATTA

SEQ. ID NO:64

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the HtrB gene from *Haemophilus influenzae* (HiRd)**

TTGAAGTCCCCAATTTACCCACCACAATTCCTGCGGCAACATTGGCTAGGTAACAAGATT  
 65 CTTCGAAAGAACGTCATCTGCTAATGTGGTTGCTAATACACTAATGACAGTGTACCCGG  
 CTCCCGTCACATCAAACACTTCTTTGCAACGGTTGGCAAATGATAAGGCTCTTGATTTG  
 GCGTAATAATGTCATGCCTTTTTCAGAACGCGTCACCAAAAGTGCAGTTAATTCATAT  
 70 CAGAAATTAATTTTAAACCTTTCTTAATAATCTTCTTCTGTATTACATTTACCTACAA  
 CGGCTTCAAATTCAGACATATTGGGTGTCAATAATGTAGCCCCACGATAACGTTCAAAT  
 CAGTTCCCTTTGGATCGATCAACACAGGCACATTGCTTTGCGTGCAATTTGAATCATTT  
 TCTGAACATCTTTAAGCGTGCCTTTGCCGTAATCAGAAAGAATCAAAGCACCGTAATTTT  
 TCACCGCACTTTCTAATTCGCTAATAAATCCTTGCAATCTACATTATTGAAATCTTCTT

CAAAATCAAGGCGGAGCAGCTGTTGATGACGAGATAAAATACGTAATTTAGTAATGGTTG  
 GATGGGTTTCTAATGCAACAAAATTACAATCAATCTTTTGTCTTCTAATAAGTGGGAAA  
 GTGCAGAACCTGTCTCATCTTGTCCAATCAATCCCATTAACTGAACGGGTACATTGAGTG  
 AAGCAATATTCATCGCCACATTTGCAGCACC GCCCGCGCTTCTTCATTTTCTTGACGC  
 5 GAACTACTGGCACTGGTGTCTGTTGAAATACGGTTGGTTGCACCGAACCAATAACGAT  
 CAAGCATCACATCGCCTAATACAAGTACTTTTGCTTGCTTAAATCTGCTGAATATTGAG  
 CCATTTTAAAATCTCTCTATTGTAATAACCAAAATTGTGGCGATTTTACCACAACCTCAA  
 TTTACGATAAACTACGCCCTAATTACGTGGAAGAACAA

10 SEQ. ID NO:65

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the protein D  
 gene from *Haemophilus influenzae* (HiRd)**

AGCAATAATTATAGCTGGAATATCTTTAAAGATGAAAGAGATCGTATAAGACAAAAAGA  
 ATTTTATATTGGAGAATTATTAGCAATTATTGGTTTCGCTAATATTCGTAATAAATAGTTC  
 15 AAATAATGATGGAAATACAGACTTTTTTCTTGGGGCAATATTTCTTTTACAGCTATTTT  
 TATTCAATCTGTACAGAATTTAATTGTAAAAAAGTAGCCAAAAGATAAATGCTGTTGT  
 AATAAGTGCATCGACAGCAACAATTTCCAGGAGTATTATTTTATGTTTAGCTTTTAATAC  
 TAAACAAAATATATTTATTACAAGATGTTGGCATTGGAATGTTGATAGGTTTAGTTGCGC  
 TGGCTTTTATGGGATGCTAACAGGGATGTTGATGGCTTTTATATTGTTCAAAAACAGGG  
 20 AATCAGCTGTTTTTAAACATTTTGAATTATTAAATTCCTCTTTCACTGCGATAATAGGTTA  
 CTTAACATTAGATGAAAGAATAAATATCTATCAGGGAATTAGCGGTATTATTGTAATTAT  
 TGGTTGTGATTGGCATTAAAAAGAAAAACAAGGAGTGTGATATATAAAGTAGATGAT  
 GTTGGTGAATAGGTATAGTTAAATATCTGGTTCAATTGGTTTTATTAAGGGCGTTAGCA  
 ATTCTCCATTTAAGTTTATGTTTGAATTAGATATTTTGGGAAAAGATGGAAGAATAAAGC  
 25 TGTTAAATAATGCTGAAACATATGAACATATACTCAATAAAAATAATTCTGCTG  
 GAAATGATTATAAATCTCTAATTTCTAATTGTAGAGAGGATAATGACTATCAATCAGAAA  
 GAATGATTAAAGCCATTAAAAATATTATTCATTGTATGACTAATAATCATCAACCTATTT  
 CAAGTGCTGAAACATCTTTAGAACTATTAAATTTATTCACGGAATAATTAAATCTGTTA  
 AAATAGGTAATGATCCTAACAATATATAAGGAGAATAAGT

30 SEQ. ID NO:66

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the Hin47 gene  
 from *Haemophilus influenzae* (HiRd)**

TAAATACTCCAAAATAAATTTTCAGATAACGTGGTCTGTAAGACAAAAAATAAAAAAAT  
 35 GTTCAATAAGAGGAGAGCAAATTATCTTGTTTAAAGGAAATCGGAGCAGTACAAAACG  
 GTCTTACAAGTAGCAAATTCTATATAGTTTCTAATACGCGCAATTTTCTAGTCAAT  
 AAAAAGGTCAAAAAATGAGCTGGATTAACCGAATTTTGTAGTAAAGTCCTTCTTCTTCCA  
 CTCGAAAAGCCAATGTGCCAGAAGGCGTATGGACAAAATGTACTGCTTGTGAACAAGTAC  
 TTTATAGTGAAGAACTCAAACGTAATCTGTATGTTTGCCCGAAATGTGGTCATCATATGC  
 40 GTATTGATGCTCGTGAGCGTTTATTAAATTTATTGGACGAAGATTCAAGCCAAGAAATTG  
 CGGCAGATTTTAGAACCAAAAGATATTTTAAATTTCAAAGATTTAAAGAAATATAAAGATC  
 GTATCAATGCGGCGCAAAAAGAAACGGGCGAGAAAGATGCGCTAATTACTATGACAGGTA  
 CACTTTATAATATGCCAATCGTTGTGGCTGCATCGAATTTGCTTTTATGGGCGGTTCAA  
 TGGGTTCTGTAGTTGGTGCAAAATTTGTTAAAGCGGCTGAAAAGCGATGGAATGAATT  
 45 GTCCATTTGTGTGTTTCTCTGCGAGTGGTGGTGCTCGTATGCAGGAAGCATTTATCTCTT  
 TAATGCAAATGGCAAAAACCTAGTGCCGTAATTTGCTCAAATGCGTGAAAAGGGTGTGCCAT  
 TTATTTTCAGTATTAACGGATCCGACTTTAGGCGGCGTATCAGCCAGTTTTCGATGTTAG  
 GGGATTTAAATATTGCCGAGCCAAAAGCCTTAATTGGTTTTGCAGGGCCACGCGTTATTG  
 50 AACAACTGTGCGTGAAAATTTGCCAGAAGGTTTCCAACGTAGTGAGTTTCTACTTGAGA  
 AAGGGGCAATTGATATGATCGTGAAACGTTTCAGAAATGCGT

SEQ. ID NO:67

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the P5 gene from  
*Haemophilus influenzae* (HiRd)**

TCACTTAATTCAAGCGCATCAATGTTTTCTAAAACATCAACAGAATTGACCGCACTTGTA  
 55 TCTAAAATTTTCGCCATTTATTAAGACTGCGCGTAATGCCAAAACATGATTAGAGGTTTTA  
 CCATATTGCAATGAGCCTTGCCAGAGGCATCGGTGTTAATCATTCCACCTAAAGTCGCT  
 CGATTGCTGGTGGACAGTTCTGGGGCAAAGAACAAACCATGTGGTTTTAAAAATTGATTA  
 AGTTGATCTTTTACTACGCCTGCTTGTACTCGAACCCAACGTTCTTTTACATTGAGTTCT  
 60 AAGATGGCTGTATATGACGAGAAAGATCCACTATTATATTGTTATTGATGGATTGCCCA  
 TTTGTGCCAGTGCCCTCCACCGGAGCGTAAAGCTGATTGATTGATATTCAGGTAAATTT  
 GCCAATTTTGTATTCCGCACTATATCAGCAACCGTTTTTCGGAAAAGAAATTGCTTGTGGA  
 AGTTGTTGGTAAACGCTGTTATCCGTAGCCAGACTTAATCTATCTGCATAGTTTGTGCGA  
 ATATCCCCCTCAAAATGTTGGCATTGAAGATCATCAAGATAATCAAGTACATATTGTTCA  
 65 ACTTGAGGAATGCGATTTAGATTTGGCAACATAGTATTTGACCCATTTAAACATATCAGA  
 TGGAGGCTTTGATAATATCCTAAGGCTAGAATAATGTCGATTAGGAAAAGAGAGAGGAGAA  
 AGTAAAAAGTCTGTTTAAAGAAAGTGTATTTTGGATAAAAACTAAACAAAAAATTCAAAA  
 GAATTTGATCTTTTCAATTTTATAGGATAATAAGCGCACTTTTGAACGTTCTTTGGGG  
 70 TAAACATAAGCAAAAGGAATTGAATTTGTCAAAGGTAATAAAGTAGGGCAAATTCAAAAC  
 CCTAGTTAAGTGACTGTTTATAATGTAGCTTTAATTAAAAGTTCAGTATAAACAAGGACA  
 CTTTTTATTACTATTCGATCACTAAATAGAGGACATCAAAA

SEQ. ID NO:68

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the D15 gene from *Haemophilus influenzae* (HiRd)**

TCGATTGTATCCTATATAAATTATAGACGTAAAAAATCATTAAATAATGCAAACACCGTT  
 5 AAGCTTAATAACAGTGCTGCGCCAATTCGATAACAGATGCTTTGCACCCGCTCAGAAACA  
 GGTTCCTTTAACAGCTTCCATTGTTAAAAAACTAAATGACCGCCATCTAATACTGGT  
 AATGGAAATAAATTCATAATCCCTAAATTTACACTAATCAATGCCATAAACTTAAAAAA  
 TACACCAATCCAATATTTGCTGATGCGCCAGCACCTTTTGCAATAGAAAATTGGCCCACTT  
 AAATTATTTAATGACAAATCGCCAGTAAGTAATTTCCCTAATATTTTCAAGGTTAAAAGG  
 10 GAAAGCTGTCCTGTTTTTCAATGCCTTTTTGTAAAGATTCAAGAATACCATATTTTAAT  
 TCAGTACGGTATTCATCCGCTAATTTTGTAAAGCTGGGCTAACCCCAACAAACCATTG  
 CCATTTTGATTACGCACTGGAGTTAGGACTTTGTCAAATGTTTCTCCATTACGTTCAACT  
 TTAATAGAAAAAGATTTCGCCTTGTTGACCTGTTTATAAAATCTTGCCAAGGAAGTGCG  
 GTTAAATTTTCTTTTAAATTTTATCACCGATTGTAAACCAGCTTCTCAGCGGGAGAA  
 15 TTTTGAACAACCTTAGAAAGCACCATTTCATTTTAGGACGCATAGGCATAATCCCTAAT  
 GCCTCAAAAGCACTTCTT'TTTTCAGGATCGAATGTCCAATTTGTAAGATTTAAAGTCCGT  
 TGTGTTCATATTAGAATTGAAAGGAGAAAGGCTAATCTCAACATTAGGCTCCCCCAT  
 TTTGTGGCAAGTAGCATATTGATGGTTTCCCAATCTTGAGTTTCTTCGCCATCAATTGTA  
 AGAATTTGCGTATTGGGTTCAATGTGGGCTTGCTGCGATTGAGTTTGGTGTATTGAT  
 20 TCAATCACTGGTTTAACCGTTGGCATTCCATAAAGGTAAAT

SEQ. ID NO:69

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the Omp26 gene from *Haemophilus influenzae* (HiRd)**

TTTGATAAATATCCTTAATTAATGATGGGTTTAATATTTTCTCTGCCCAATTAAATTAG  
 25 GCAGAGAACGTTGTTTTGAGTTCTGATGAAGAAAAAGTTCAATTTATTAGAAAGAACC  
 TCCAATACTAAATTGGAACGTTCGACATCATTTTCATATTTTAAATTGGTTTGGC  
 ATAAGAGAATAACCAATGGCCCAATAGGAGATTGCCATTGGAATCCGACACCTGTAGAGGC  
 GCGAATACGGCTTGATTGGCATAATCGGGTAAGCTTTTTAATACATTGTTATCTAACCC  
 ACTCTTATCCGATTCCACTTAGTATTCACAACTTGCCGCATCAACAAATAGGGAGGT  
 30 TCGGACTGTATTTGGCTTTTATCACTCACAAACGGTGTGGTACAATAAGTTCTGCACT  
 CGCAGTTGTGATTGCATTACCACCAATCACATCAGAACTTATCTTCTTAAAAGTACCATT  
 ACCATTACCATGTTCTGCATAAATTGCGTTAGGTCCAATACTACCATAAGCAAACACG  
 TAATGAACCGATGCCACCCGCTGTATAAGTTTGATAGAACGGTAAACGCTTGTTCCTAAA  
 ACCATTTGCATATCCTGCGAGATGCTTTTGAGATACAACCCAGAGGTGATCTCTGTCTAA  
 35 TGGGTAGAAACCCGTGACGTCTGCTTTGTAGTATTTGTTATCAGAACCCTGGAAT  
 AGTAACCTCGTCCACCAAGACTTGCTTTAACCCTTTAGTTGGGAAATAGCCTCTATTAAG  
 GCTGTTATAGTTCCAACCAAAAGAAAAATCAAAGTCATTTGTTTAAATGCCATTACCTTT  
 AAATTTTCATTGATTGAATATATAAATTACGGTTATATTCTAGAGCAAAGTTACTAATTTT  
 40 ATTATAGGTATGGCCTAATCCTACATAATAGGAGTTATTTTCATTTACAGGGAAACCTAA  
 AGTAACATTACTTCCATAAGTCGTACGCTTATAGTTAGAGG

SEQ. ID NO:70

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the P6 gene from *Haemophilus influenzae* (HiRd)**

TTAGATTTCTCCTAAATGAGTTTTTTATTTAGTTAAGTATGGAGACCAAGCTGGAAATTT  
 45 AACTTGACCATCACTTCCGGAAGGCTCGCCTTAAAGCGACCATCTGCGGAAACCAATTG  
 TAGCACCTTTTCTAAGCCCTGTGTAGAACTATAAATAATCATAATTCCATTGGAGAGAG  
 GCTTGGGCTTTTCGCTAGAAAAGATGTACTAAGTACCTCTGAAACGCCCGTTGTGAGATC  
 TTGTTTAACTACATTATTGTTACCATTAAATCATCACAAGTGTTTTTCCATCTGCACTAAT  
 50 TTGTGCGCTACCGCGACCACCCACTGCTGTGCACTACCACCGCTTGCATCCATTTCGATA  
 AACTTGTGGCGAACCATTCTATCGGATGTAATAAATTGAATTTCCGTCTGGCGACCA  
 CGCTGGTTTCAGTATTATTACCCGCAACCATCGTCAATTGAGTAGGTGTACCGCCATTTGC  
 TCCCATAAACGTAAATATTCAGAACCCATCACGAGAAGAAGCAAAAGCTAAACGAGAACC  
 ATCTGGCGAAAAGGCTGGTGCGCCATTATGCCCTTGAAAAGATGCCACTACTTTACGTGC  
 55 GCCAGAAATTTAAATCCTGTACAACAAGTTGTGATTTTTTATTTTCAAACGATACATAAGC  
 CAAACGCTGGCCGTCTGGAGACCAAGCTGGAGACATAATTGGTTGGGCACTACGATTGAC  
 GATAAATTGATTATAGCCATCATAATCTGCTACACGAACCTTCATAAGGTTGCGAACCAGCC  
 ATTTTTTTGCACAACATAAGCGATACGAGTTCTAAAGGCACACGGATCGCAGTTAATTT  
 TTCAAAAACCTTCATCGCTCACAGTATCGCGCCATAGCGTAACCATTATTTGTTACTGT  
 60 ATAGCTATTTTGCATTAAATACAGTCCCTGGCGTACCTGATGCACCAACCGTATCAATTAA  
 TTGATAAGTAATACTATAACCATTACCCGATGGAACCACTT

SEQ. ID NO:71

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the TbpA gene from *Haemophilus influenzae* (non-typeable)**

GGCGATAACCGAGTTTTTGGGGTATTAGTGCCAAAGAAGACCCACAAAACCCAAAATTA  
 65 TCCAGAGAAACCTTAATTGATGGCAAGCTAACTACTTTTAAAGAAGTATGCAAAAACCC  
 AATACAACAGCCGATACAACAACCAATAAAACAACCAATGCAATAACCGATGAAAAAAC  
 TTTAAGACGGAAGATATACTAAGTTTTGGTGAAGCTGATTATCTTTAATTGACAATCAG  
 70 CCTGTTCCGCTTTTACCTGAAAAAATACTGATGATTTTATAAGTAGTAGGCATCATACT  
 GTAGGAAATAAACGCTATAAAGTGAAGCATGTTGCAAGAATCTAAGCTATGTAAATTT  
 GGTATGTATTATGAAGACCCACTTAAAGAAGAAGAAAAAGAAAAAGAAAAAGAC  
 CAAGAAAAAAGAAAAAGAAAAACAAACGACGACAACATCTATCGAGACTTATTATCAA  
 TTCTTATTAGGTCACCGTACTGCCAAGGCCGACATACCTGCAACGGGAAACGTGAAATAT



CGCGGTAATTGGTTTGGTTATATTGGTGATGACACGACATCTTACTCCACTACTGGAGAT  
 AAAAATGCTCTCGCCGAGTTTGATGTAAATTTTGCCGATAAAAAGCTAACAGGCGAATTA  
 AAACGACACGATAATGGAAATACCGTATTTAAAATTACTGCAGACCTTCAAAGTGGTAAG  
 AATGACTTCACTGGTACAGCAACCGCAACAAATTTTGTAATAGATGGTAACAATAGTCAA  
 5 ACTGGAAATACCCAAATTAATATTAATACTGAAGTAAATGGGGCATTATTTATGGACCTAAG  
 GCTACAGAATTAGGCGGTTATTTACCTATAACGGAAATTCTACAGCTAAAAATTCCTCA  
 ACCGTACCTTCACCACCCAATTACCAAATGCAAGAGCTGCAGTTGTGTTTGGAGCTAAA  
 AAACAACAAGTAGAAACAACCAAGTAATGGAATACTAAAA

10 SEQ. ID NO:72

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the TbpB gene from *Haemophilus influenzae* (HiRd)**

TAGAATTATATTCTTATACAAAATTGATAATTGTTTCGCATTATCATTTTTTTTTTGTAAAT  
 AATGTCAACTTATAATTTTTTAAGTTCATGGATAAAATATGAAAAATGGCGTAAAAACAAC  
 15 TTTTCTCTTATCATTAATAGGCTTATCATTAACGAATGTAGCTTGGGCAGAAGTTGCAC  
 GTCCATAAAATGATACATTGACAAATACCAATCAAAGTGCAGGAATTAACCACTCCTCTT  
 TTTCTCTATGCCTAAGAAAGAAATACCAATAGGCATATTATTTCTCTTTCCAAAAGCC  
 AATTAGCGCACCATCCAAGGCTTGTTTTGCGTGGGTTAATTCCTGCTTTATATCAAAAATA  
 ACACTCAGGCAGTTCAACTGTTATTACCACTATATAACAATTCCTCAACAAGATAATT  
 20 TCTTACTAAGTTGGGCAAAGGCTATTGAAGCTCGTGAACAAGGTGATTTAACTCAATCTA  
 TTGCTTATTATCGTGAATTATTGCTCGAGACGCATCTTACTACCTTTACGTTATTAAT  
 TAGCTCAAGCTCTATTTTTTAACTATGAAAATGAAGCTGCCAAAATTCATTTGAAAAAT  
 TACGTACAGAGGTAGATGATGAAAAATTTTAGGTGTTATTGATCAGTATCTTTTAACAC  
 TAAATCAGCGGAATCAATGGATATGGCAAGTAGGATTAAATTTTTTAAATGATGATAATT  
 25 TGAATAACGCTCCAAAAGTGGCACAATAATGGTAGTTGGACCGCTTGGGAAAAAGAAA  
 GTGGGCAGGGGGTAGGGTATTCTTTATCAGTAGAAAAAAATGGCCATGGGCAGATCATTT  
 TTTTGTAGTAAACTATGTTTAAATGGGAATGGAAAATATTATTGGGATAATAAAAAATACA  
 ATGAGGCTACTGTGCGTATAGGTGGTGGTTFAGGCTATCAAACCTGCCTCAGTTGAAGTCT  
 CGTTGTTTCCTTTTCAAGAAAAACGCTGGTATGCAGGCGGT

30 SEQ. ID NO:73

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the HifA (pilin) gene from *Haemophilus influenzae* (LKP serotype 1 genome)**

TAATAAATTGCTCCATAAAGAGGTTTGTGCCTTATAAATAAGGCAATAAAGATTAAATATA  
 35 AACCGTTTATTAATAATGCCAAAGGCTTAATAACAGCAAACTTTGTTTTCCCAAAAAAAG  
 TAAAAAATCTTCCATTATATATATATATATATAATTAAGCCCTTTTTGAAAAATTT  
 CATATTTTTTTGAATTAATTCGCTGTAGGTTGGGTTTTTGCCACATGGAGACATATAAA  
 AAAGATTTGTAGGGTGGGCGTAAGCCACGCGGAACATCATCAACAACGTGAATGTTGT  
 ATTAGGCACGGTGGGCTTATGCCTCGCCTACGGGGAAATGAATAAGGATAAATATGGGCT  
 40 TAGCCAGTTTATGGATTTAATTATGTTGAAATGGGGAAAAACAATGTTAAAAAAACACT  
 TTTATTTTTTACCGCACTATTTTTTGCCGCACCTTTGTGCATTTTCAGCCAATGCAGATGT  
 GATTATCACTGGCACCAGAGTGATTTATCCCGCTGGGCAAAAAAATGTTATCGTGAAGTT  
 AGAAAACAATGATGATTCGGCAGCATTTGTGCAAGCCTGGATTGATAATGGCAATCCAAA  
 TGCCGATCCAAAATACACCAAAACCCCTTTTGTGATTACCCCGCCTGTTGCTCGAGTGGA  
 45 AGCGAAATCAGGGCAAAGTTTGCAGATTACGTTACAGGCAGCGAGCCTTTACCTGATGA  
 TCGCGAAAGCCTCTTTTATTTTAAATTTGTTAGATATTCCGCCGAAACCTGATGCGGCATT  
 TCTGGCAAACACGGCAGCTTTATGCAAATTGCCATTGCTCAGTTTGAAGTTGTTTAA  
 TCGCCCTGCGAAACTCTCGATGGATTCTCGTGATGCAATGAAAAAGTAGTGTTTAAAGC  
 CACACCTGAAGGGGTGTGGTGGATAATCAAACCCCTTATTATATGAATACATTGGTTT  
 50 GTTACATCAAAATAAACCTGCGAAAAATGTCAAAATGGTTG

SEQ. ID NO:73

**Nucleotide sequence of DNA region (1000 bp ) up-stream from the HifE (tip pilin) gene from *Haemophilus influenzae* (LKP serotype 1 genome)**

TAGTAGATTTCCGCACGGGCAAAAATACAATGGTGTATTTAACCTCACTTTGCCAAATG  
 GCGAGCCAGTGCCAAATGGCATCCACCGCACAAAGATAGCGAAGGGGCATTTGTGGGCGATG  
 TGGTGCAAGGTGGTGTGCTTTTCGCTAATAAACTTACCCAGCCAAAAGGCGAGTTAATCG  
 TCAAATGGGGTGAGCGAGAAAGCGAACAATGCCGTTTCCAATATCAAGTTGATTTGGATA  
 60 ACGCACAAATACAAAGTCACGATATTCAATGCAAAACCGCAAAATAAATAATTGAAGAGG  
 ATTTATGCAAAAAACCCCAAAAATTAACCGCGCTTTTCCATCAAAAATCCACTGCTAC  
 TTGTAGTGGAGCAAATTATAGTGGAGCAAATTATAGTGGCTCAAAATGCTTTAGGTTTCA  
 TCGTCTGGCTCTGCTTGCTTGCGTGGCTCTGCTTGATTGCATTGTGGCACTGCCTGCTTA  
 TGCTTACGATGGCAGAGTGACCTTTCAAGGGGAGATTTTAAGTGTGGCACTTGTAATAAT  
 TGAAACAGACAGCCAAAATCGCACGGTTACCTGCCAACAGTGGGAAAAGCTAATTTAAG  
 65 CCACGCAGGGCAAACCGCGCCCTGTGCCTTTTCCATCACGTTAAAAGAATGCAATGC  
 AGATGATGCTATGAAAGCTAATCTGCTATTTAAAGGGGGAGACAACACAACAGGGCAATC  
 TTATCTTTCCAATAAGGCAGGCAACGGCAAGCCACCAACGTGGGCATTCAAATTGTCAA  
 AGCCGATGGCATAGGCACGCCTATCAAGGTGGACGGCACCAGCCAACAGCGAAAAAGC  
 CCCCAGACAGGTAAAGCGCAAAACGGCACAGTTATTCAACCCCGTTTTGGCTACTTTGG  
 70 CTCGTTATTACGCCACAGGTGAAGCCACCGCAGGCGACGTTGAAGCCACTGCAACTTTTG  
 AAGTGCAGTATAACTAAAATATTTATATCCAGTGAAAAA

SEQ. ID NO:75



**Nucleotide sequence of DNA region (1000 bp ) up-stream from the P2 gene from *Haemophilus influenzae* (HiRd)**

```

1  TTATCCGCTA ACATTTTCATC AGTAATTCCA TGAAC TTTAA TCGCATCAGG
51 ATCANCGGGG CGATCTGGCT TAATATAAAT ATGAYAATTA TTACCTGTGT
5  101 AACGACGATT TATTAATTCA ACTGCACCAA TTTCAATAAT GCAGTGTCCCT
151 TCATAATGCG CGCCAAGCTG ATTCATACCT GTAGTTTCAG TATCTAATAC
201 AATTTGGCGA TTGGGATTAA TCATTTGTTC AACCTATCTC TTTCCATTAA
251 AATACTTGCC ATTCTACACA ACAACCTTTT TGTATGCK AAACAGATTG
301 AAATTTTAC TGATGGATCT TGCTTAGGTA ATCCAGGGGC GGGCGGAATT
10 351 GGTGCCGTAT TGCGTTATAA ACAACATGAA AAAACACTCT CCAAAGGCTA
401 TTTCCAAACC ACCAATAATC GAATGGAATT ACGCGCTGTC ATTGAAGCAT
451 TAAATACATT AAAAGAACCT TGCTTGATCA CGCTTTATAG TGATAGCCAA
501 TATATGAAAA ATGGCATAAC CAAATGGATC TTTAACTGGA AAAAAAATAA
551 TTGGAAAGCA AGTTCTGGAA AGCCTGTAAA AAACCAAGAT TTATGGATAG
15 601 CCTTAGATGA ATCCATCCAA CGTCATAAAA TTAATTGGCA ATGGGTAAAA
651 GGCCATGCTG GACACAGAGA AAATGAAATT TGCGATGAAT TAGCAAAAAA
701 AGGGGCAGAA AATCCGACAT TGGAAGATAT GGGGTACATA GAAGAATAAT
751 ACAACTGATA TAACGTCATA TTTTTCGATA CCTAAAAATA TTTAATACTT
801 AAACCTAAAA CAGAATAAAA AATAATCAAA TTCATTTAAA AAATGTGATC
20 851 TCGATCAGAT TTCAAGAAAA TTAAAATTTT GGAGTATTGA CATCAAAAAT
901 TTTT TTTGTA AAGATGCAGC TCGTCCGTTT TGGCGATTGG ACAATTCTAT
951 TGGAGAAAAG TTCAATCATA GATAGTAAAC AACCATAAGG AATACAAATT
1001 A

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25 SEQ. ID NO:76

**Nucleotide sequence of DNA coding region (partial) of the *Moraxella Catarrhalis* HtrB gene**

```

1  TCAGTGCTTG GTTTTTTAAG ATATGTACCG CTGTCAGTCC TGCATGGATT
51 GCGGGCGTGT GCGTCTTATA TTTCCATCA TTGCAGGCTT AGTATTTATC
30 101 GCAGCATCCA AGCCAATTTA ATCTTGGTTC ACCCCAAGAT GCCAGACGCA
151 CAGCGGCAAA AACTCGCCAA ACAAATCCTA AAAAATCAGC TCATCAGTGC
201 AGTCGACAGT CTTAAAACCT GGGCAATGCC ACCAAAATGG TCTATCGCAC
251 AAATTAAAC GGTTCATCAT GAAGATATCC TAATCAAAGC ACTTGCCAAT
301 CCAAGTGGTA TGCTTGCCAT TGTGCCCTCAT ATCGGCACTT GGGAGATGAT
35 351 GAATGCTTGG CTCAATACCT TTGGCTCCCC TACTATCATG TATAAGCCCA
401 TCAAAAATGC GCGGGTAGAT CGCTTTGTTT TACAGGGGCG TGAAAGACTA
451 AATGCCAGCC TTGTACCCAC AGATGCTAGT GGTGTTAAGG CAATTTTAA
501 AACACTCAA GCAGGTGGAT TTAGTATCAT ACTGCCCGAC CATGTACCTG
551 ATCCATCAGG TGGTGAGATT GCTCCTTTTT TTGGTATTAA AACCTAACC
40 601 AGTACGCTGG CGTCAAAGCT TGCTGCAAAA ACTGGTTGTG CTCTTGTGG
651 CTTAAGCTGT ATTCGGCGTG AAGATGGCGA TGGTTTTGAA ATTTTGTGTT
701 ATGAATTAAA TGATGAACAA CTTTATTCAA AAAATACCAA AATTGCAACC
751 ACTGCTTTAA ATGGTGGCAT GGAACAAATG ATTTATCCAC ATTTTGTGCA
801 TTATATGTGG AGCTATCGTC GGTTCAGCA TACACCACTA TTAAATAATC
45 851 CTTATTTACT TAATGAAAT GAGCTAAAAA AAATAGCCAT AAAGCTTCAA
901 GCCATGTCAA AGGATAGTTA TGAG

```

Protein Seq: 25% identity and 35% similarity with HtrB from *E. coli*

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1  SVLGFLRYVP LSVLHGLAAC ASYISYHCRL SIYRSIQANL ILVHPKMPDA
50 51 QRQKLAKQIL KNQLISAVDS LKTWAMPPKW SIAQIKTVHH EDILIKALAN
101 PSGMLAIVPH IGTWEMMNAW LNTFGSPTIM YKPIKNAVD RFVLQGRERL
151 NASLVPTDAS GVKAFKTLK AGGFSIILPD HVPDPGGEI APFFGIKTLT
201 STLASKLAAK TGCAVLGLSK IRREDGDGFE IFCYELNDEQ LYSKNTKIAT
251 TALNGAMEQM IYPHFLHYMW SYRRFKHTPL LNNPYLLNEN ELKKIAIKLQ
55 301 AMSKDSYE

```

SEQ. ID NO:77

**Nucleotide sequence of DNA coding region of the *Neisseria* (meningococcus B)**

```

60 1  ATGTTTCGTT TACAATTCGG GCTGTTTCCC CTTTTCGAA CCGCCATGCA
51 CATCCTGTTG ACCGCCCTGC TCAAATGCCT CTCCCTGCTG CCACTTTCCT
101 GTCTGCACAC GCTGGGAAAC CGGCTCGGAC ATCTGGCGTT TTACCTTTTA
151 AAGGAAGACC GCGCGCGCAT CGTCGCCAAT ATGCGTCAGG CAGGCATGAA
65 201 TCCCGACCCC AAAACAGTCA AAGCCGTTTT TGCGGAAACG GCAAAAGGCG
251 GTTTGGAAC TGCCTCCGCG TTTTTCAGAA AACCAGGAAGA CATAGAAACA
301 ATGTTCAAAG CGGTACACGG CTGGGAACAT GTGCAGCAGG CTTTGGACAA
351 ACACGAAGGG CTGCTATTCA TCACGCCGCA CATCGGCAGC TACGATTTGG
401 GCGGACGCTA CATCAGCCAG CAGCTTCCGT TCCCGCTGAC CGCCATGTAC
70 451 AAACCGCCGA AAATCAAAGC GATAGACAAA ATCATGCAGG CGGGCAGGGT
501 TCGCGGCAAA GGAAAAACCG CGCCTACCA CATAAAGGG GTCAAACAAA
551 TCATCAAAGC CCTGCGTTTG GCGGAAGCAA CCATCGTCCT GCCCGACCAC
601 GTCCCTTCCC CTCAAGAAGG CGGGGAAGGC GTATGGGTGG ATTTCTTCGG
651 CAAACCTGCC TATACCATGA CGCTGGCGGC AAAATTGGCA CACGTCAAAG

```

701 GCGTGAAAAC CCTGTTTTTC TGCTGCGAAC GCCTGCCTGG CGGACAAAGGT  
751 TTCGATTTGC ACATCCGCCC CGTCCAAGGG GAATTGAACG GCGACAAAGC  
801 CCATGATGCC GCGGTGTTCA ACCGCAATGC CGAATATTGG ATACGCCGTT  
851 TTCCGACGCA GTATCTGTTT ATGTACAACC GCTACAAAAT GCCG

5

Protein Sequence - 30% identity and 38% similarity with Htrb *E. coli*

1 MFRLQFGLFP PLRTAMHILL TALLKCLSLP PLSCLHTLGN RLGHLLAFYLL  
51 KEDRARIIVAN MRQAGMNPDP KTVKAVFAET AKGGLELAPA FFRKPEDIET  
101 MFKAHVHWEH VQQALDKHEG LLFITPHIGS YDLGGYISQ QLPFPLTAMY  
151 KPPKIKAIK IMQAGRVRGK GKTAPTSIQG VKQIIKALRS GEATIVLPDH  
201 VPSPQEGGEG VVVDFFGKPA YTMTLAAKLA HVKGVKTLFF CCERLPGGQG  
251 FDLHIRPVQG ELNGDKAHD AVEFNRAEYW IRRFPTQYLF MYNRYKMP

10

SEQ. ID NO:78

15 Nucleotide sequence of DNA coding region of the Haemophilus influenzae (non-typeable) HtrB gene

1 ATGAAAAACG AAAAAGTCCC TCAATTTCAA CCGCACTTTT TAGCCCCAAA  
51 ATACTGGCTT TTTTGGCTAG GCGTGGCAAT TTGGCGAAGT ATTTTATGTC  
101 TTCCCTATCC TATTTTGC GC CATATTGGTC ATGGTTTCGG TTGGCTGTTT  
20 151 TCACATTTAA AAGTGGGTAA ACGTCGAGCT GCCATTGCAC GCCGTAATCT  
201 TGAACCTTGT TTCCCTGATA TGCCCTGAAAA CGAACGTGAG ACGATTTTGC  
251 AAGAAAATCT TCGTTCAGTA GGCATGGCAA TTATCGAAAC TGGCATGGCT  
301 TGGTTTTGGT CGGATTCACG TATCAAAAAA TGGTCGAAAG TTGAAGGCTT  
351 ACATTATCTA AAAGAAAATC AAAAAGATGG AATTGTTCTC GTCGGTGTTC  
25 401 ATTTCTTAAC GCTAGAACTT GGCGCACGCA TCATTGGTTT ACATCATCCT  
451 GGCATTGGTG TTTATCGTCC AAATGATAAT CCTTTGCTTG ATTGGCTACA  
501 AACACAAGGC CGTTTACGCT CCAATAAAGA TATGCTTGAT CGTAAAGATT  
551 TACGCGGAAT GATCAAAGCT TTACGCCACG AAGAAACCAT TTGGTATGCG  
601 CCTGATCACG ATTACGGCAG AAAAAATGCC GTTTTTGTTC CTTTTTTTGC  
30 651 AGTACCTGAC ACTTGCACTA CTAAGGTAG TTATTATTTA TTGAAATCCT  
701 CGCAAAACAG CAAAGTGATT CCATTTGCGC CATTACGCAA TAAAGATGGT  
751 TCAGGCTATA CCGTGAGTAT TTCAGCGCCT GTTGATTTTA CGGATTTACA  
801 AGATGAAACG GCGATTGCTG CGCGAATGAA TCAAATCGTA GAAAAGGAAA  
851 TCATGAAGGG CATATCACAA TATATGTGGC TACATCGCCG TTTTAAAACA  
35 901 CGTCCAGATG AAAATACGCC TAGTTTATAC GATTAA

35

Protein Sequence - 57% identity and 66% similarity with HtrB *E. coli*

1 MKNEKLPQFQ PHFLAPKYWL FWLGVAIWR ILCLPYPILR HIGHGFGWLF  
51 SHLVKGKRR AIARRNLELC FPDMPENERE TILQENLRV GMAIETGMA  
40 101 WFWSDSRIKK WSKVEGLHYL KENQKDGIVL VGVHFLTLEL GARIIGLHHP  
151 GIGVYRPNDN PLLDWLQTOG RLRSNKDMLD RKDLRGMKA LRHEETIWYA  
201 PDHDYGRKNA VFVPFFAVPD TCTTTGSYYL LKSSQNSKVI PFAPLRNKDG  
251 SGYTVSISAP VDFTDLQDET AIAARMNQIV EKEIMKGISQ YMWLHRRFKT  
301 RPDENTPSLY D\*

45

SEQ. ID NO:79

Nucleotide sequence of DNA coding region of the Haemophilus influenzae (non-typeable) MsbB gene

1 ATGTCGGATA ATCAACAAAA TTTACGTTTG ACGGCCAGAG TGGGCTATGA  
50 51 AGCGCACTTT TCATGGTCGT ATTTAAAGCC TCAATATTGG GGGATTTGGC  
101 TTGGTATTTT CTTTTTATTG TTGTTAGCAT TTGTGCCTTT TCGTCTGCGC  
151 GATAAAATTGA CGGAAAATT AGGTATTTGG ATTGGGCATA AAGCAAAGAA  
201 ACAGCGTACG CGTGCACAAA CTAACCTGCA ATATTGTTTC CCTCATTTGA  
251 CTGAACAACA ACGTGAGCAA GTGATTGATA AAATGTTTGC GGTGTGCGCT  
55 301 CAGGTTATGT TTGGTATTGG TGAGATTGCC ATCCGTTCAA AGAAACATTT  
351 GCAAAAACGC AGCGAATTTA TCGGTCTTGA ACATATCGAA CAGGCAAAAG  
401 CTGAAGGAAA GAATATTATT CTTATGGTGC CACATGGCTG GCGGATTGAT  
451 GCGTCTGGCA TTATTTTGCA CACTCAAGGC ATGCCAATGA CTTCTATGTA  
501 TAATCCACAC CGTAATCCAT TGGTGGATTG GCTTTGGACG ATTACACGCC  
60 551 AACGTTTCGG CGGAAAATG CATGCACGCC AAAATGGTAT TAAACCTTTT  
601 TTAAGTCATG TTCGTAAAG CGAAATGGGT TATTACTTAC CCGATGAAGA  
651 TTTTGGGGCG GAACAAAGCG TATTTGTTGA TTTCTTTGGG ACTTATAAAG  
701 CGACATTACC AGGGTTAAAT AAAATGGCAA AACTTTCTAA AGCCGTTGTT  
751 ATTCCAATGT TTCCTCGTTA TAACGCTGAA ACGGGCAAAT ATGAAATGGA  
65 801 AATTCATCCT GCAATGAATT TAAGTGATGA TCCTGAACAA TCAGCCCGAG  
851 CAATGAACGA AGAAATAGAA TCTTTTGTTA CGCCAGCGCC AGAGCAATAT  
901 GTTTGGATT TGCATTTATT GCGTACAAGG AAAGATGGCG AAGATCTTTA  
951 TGATTAA

70

Protein Sequence - 45% identity and 56% similarity with MsbB *E. coli*

1 MSDNQQLRL TARVGYEAF SWSYLPQYW GIWLGIFLL LLAFVPFRLR  
51 DKLTGKLGW IGHKAKKQRT RAQTNLQYCF PHWTEQQREQ VIDKMFVVA  
101 QVMFGIGEIA IRSKKHLQKR SEFIGLEHIE QAKAEGKNII LMVPHGWAI  
151 ASGIILHTQG MPMTSMYNPH RNPLVDWLWT ITRQRFGGKM HARQNGIKPF  
75 201 LSHVRKGEMG YYLPDEDFGA EQSVFVDFFG TYKATLPGLN KMAKLSKAVV  
251 IPMEPRYNAE TGKYEMEIH AMNLSDDPEQ SARANNEIE SFVTPAPEQY

75

301 VWILQLLRTR KDGEDLYD\*

SEQ. ID NO:80

Nucleotide sequence of DNA coding region of the *Moraxella catarrhalis* MsbB

5 gene

```

1  ATGAGTTGCC ATCATCAGCA TAAGCAGACA CCCAAACACG CCATATCCAT
51 TAAGCATATG CCAAGCTTGA CAGATACTCA TAAACAAAGT AGCCAAGCTG
101 AGCCAAAATC GTTTGAATGG GCGTTTTTAC ATCCCAAATA TTGGGGAGTT
151 TGGCTGGCTT TTGCGTTGAT TTTACCGCTG ATTTTCTAC CGCTGCGTTG
10 201 GCAGTTTGG ATCGGCAAGC GTCTTGGCAT TTTGGTACAT TACTTAGCTA
251 AAAGCCGAGT TCAAGACACT CTAACCAACC TGCAGCTTAC CTTCCCAAAT
301 CAACCAAAAT CAAAACACAA GGCCACCGCA CGGCAAGTAT TTATTAATCA
351 AGGTATTGGT ATTTTGTAAA GTTTATGTGC ATGGTTTCGC CCTAATGTCT
401 TTAACGCAC TTTTAGCATT TCTGGTTTAC AGCATTTGAT TGATGCCCAA
15 451 AAACAAAATA AAGCGGTGAT TTTACTTGGT GGACATCGCA CGACGCTTGA
501 TTTGGGCGGT CGGTTATGTA CACAGTTTTT TGCGGCGGAC TCGGTGTATC
551 GCCCACAAA CAACCCTTTG CTTGAATGGT TTATCTATAA TGCACGCCGC
601 TGTATCTTTG ATGAGCAAAT CTCAAATCGT GATATGAAAA AACTCATCAC
651 TCGGCTCAAA CAAGGTCGGA TAATTTGGTA TTCACCTGAT CAAGATTTTG
20 701 GTCTTGAGCA TGGCGTGATG GCGACCTTTT TTGGTGTGCC TGCAGCAACG
751 ATTACCGCTC AGCGTCGTCT TATTAAGCTG GGTGATAAAG CCAATCCTCC
801 TGTCAATCATC ATGATGGATA TGCTCAGACA AACGCCCGAT TATATCGCAA
851 AAGGTCACCG TCCACATTAT CACATCAGCC TAAGCGCTGT GTTAAAAAAT
901 TATCCCAGCG ATGACGAAAC CGCCGATGCT GAACGCATCA ATCGACTGAT
25 951 TGAGCAAAAT ATTCAAAAAG ATTTAACCCA GTGGATGTGG TTTCATCGCC
1001 GCTTTAAAC TCAAGCCGAT GACACCAATT ACTATCAACA TTAATG

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Protein Sequence - 28% identity and 37 similarity with MsbB of *E. coli*

```

1  MSCHHQHKQT PKHAISIKHM PSLTDTHKQS SQAEPKSEFW AFLHPKYWGV
30 51 WLAFALILPL IFLPLRWQFW IGKRLGILVH YLAKSRVQDT LTNLQLTFPN
101 QPKSKHKATA RQVFINGGIG IFESLCAWFR PNVFKRTFSI SGLQHLIDAQ
151 KQNKAVILLG GHRTTLDLGG RLCTQFFAAD CVYRPQNNPL LEWFIYNARR
201 CIFDEQISNR DMKKLITRLK QGRIIWYSPD QDFGLEHGVM ATFFGVPAAT
251 ITAQRRLIKL GDKANPPVII MMDMLRQTPD YIAKGHRPHY HISLSAVLKN
35 301 YPSDDETADA ERINRLIEQN IQKDLTQWMW FHRRFKTQAD DTNYYQH*

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SEQ. ID NO:81

Nucleotide sequence of DNA coding region of the *Neisseria* (meningococcus B)

MsbB gene

```

40 1  ATGAAATTTA TATTTTTTGT ACTGTATGTT TTGCAGTTTC TGCCGTTTGC
51  GCTGCTGCAC AAAGTTGCCG ACCTGACGGG TTTGCTCGCC TACCTTTTGG
101 TCAAACCCCG CCGCCGTATC GCGGAAATCA ATTTGGCAAA ATGCTTTCCC
151 GAGTGGGACG GAAAAAGCG CGAAACCGTA TTGAAGCAGC ATTTCAAACA
201 TATGGCGAAA CTGATGCTTG AATACGGCTT ATATTGGTAC GCGCCTGCCG
45 251 GCGGTTTGAA ATCGCTGGTG CGTTACCGCA ATAAGCATTA TTTGGACGAC
301 GCGCTGGCGG CGGGGGAAAA AGTCATCATT CTGTACCCGC ACTTCACCGC
351 GTTCGAGATG GCGGTGTACG CGCTTAATCA GGATGTACCG CTGATCAGTA
401 TGTATTCCCA CCAAAAAAAC AAGATATTGG ACGCACAGAT TTTGAAAGGC
451 CGCAACCGCT ACGACAATGT CTTCTTATC GGGCGCACCG AAGGCGTGCG
50 501 CGCCCTCGTC AAACAGTTCC GCAAAAGCAG CGCGCCGTTT CTGTATCTGC
551 CCGATCAGGA TTTCGGACGC AACGATTCGG TTTTGTGGA TTTTTCGGT
601 ATTCAGACGG CAACGATTAC CGGCTTGAGC CGCATTGCCG CGCTTGCAAA
651 TGCAAAAGTG ATACCCGCCA TCCCGTCCG CGAGGCGGAC AATACGGTTA
701 CATTGCATTT CTACCCGGCT TGGGAATCCT TTCCGAGTGA AGATGCGCAG
55 751 GCCGACGCGC AGCGCATGAA CCGTTTATC GAGGAACCGT GCGCGAACAT
801 CCCGAGCAGT ATTTTGGCT GCACAAGCGT TTCAAAACCC GTCCGGAAGG
851 CAGCCCCGAT TTTTACTGAT ACGTAA

```

Protein Sequence - 25% identity and 36% identity with MsbB *E. coli*

```

60 1  MKFIFVLYV LQFLPFALLH KLADLTGLLA YLLVKPRRRI GEINLAKCFP
51  EWDGKKRETV LKQHFHMAK LMLEYGLWY APAGRLKSLV RYRNKHYLDD
101 ALAAGEKVII LYPHFTAFEM AVYALNQDVP LISMYSHQKN KILDAQILKG
151 RNRYDNVFLI GRTEGVRLV KQFRKSSAPF LYLPDQDFGR NDSVFDVDFG
201 IQTATITGLS RIAALANAKV IPAIPVREAD NTVTLHFYPA WESFPSEDAQ
65 251 ADAQRMNRFI EEPANIPSS IFGCTSVSKP VRKAAPIFTD T*

```

**We Claim:**

1. An immunogenic composition comprising an antigen derived from a pathogen  
5 which is capable of protecting a host against said pathogen, mixed with an  
adjuvant comprising a bleb preparation derived from a Gram-negative bacterial  
strain, with the proviso that an immunogenic composition consisting of *N.*  
*meningitidis* B blebs and *N. meningitidis* C polysaccharide antigen is not claimed.
- 10 2. The immunogenic composition comprising an antigen comprising 1 or more  
conjugated meningococcal capsular polysaccharides selected from a group  
comprising: A, Y or W, mixed with an adjuvant comprising a bleb preparation  
from meningococcus B.
- 15 3. The immunogenic composition of claim 1, wherein the antigen and the Gram-  
negative bacterial bleb preparation are from different pathogens.
4. The immunogenic composition of claim 3, wherein the antigen is a conjugated  
capsular polysaccharide from *H. influenzae* b, and the bleb preparation is from  
20 meningococcus B.
5. The immunogenic composition of claim 3, wherein the antigen is one or more  
conjugated capsular polysaccharide(s) from *Streptococcus pneumoniae* selected  
from the group consisting of: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14,  
25 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F, and the bleb preparation is from  
meningococcus B.
6. The immunogenic composition of claim 2, 4 or 5, wherein the bleb preparation is  
derived from a strain which has a detoxified lipid A portion of bacterial LPS, due  
30 to the strain having been engineered to reduce or switch off expression of one or  
more genes selected from the group consisting of: htrB, msbB and lpxK.



7. The immunogenic composition of claim 2, 4, 5 or 6, wherein the bleb preparation is derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to express at a higher level one or more genes selected from the group consisting of: pmrA, pmrB, pmrE and pmrF.
- 5
8. The immunogenic composition of claim 2, 4, 5, 6 or 7, wherein the bleb preparation is derived from a strain which does not produce B capsular polysaccharide, due to the strain having been engineered to reduce or switch off expression of one or more genes selected from the group consisting of: galE, siaA, 10 siaB, siaC, siaD, ctrA, ctrB, ctrC and ctrD.
9. The immunogenic composition of claim 3, wherein the antigen is from *H. influenzae*, and the bleb preparation is from *Moraxella catarrhalis*.
- 15 10. The immunogenic composition of claim 9, wherein the antigen is a conjugated capsular polysaccharide from *H. influenzae* b.
11. The immunogenic composition of claim 3, wherein the antigen is from *Streptococcus pneumoniae*, and the bleb preparation is from *Moraxella 20 catarrhalis*.
12. The immunogenic composition of claim 11, wherein the antigen is one or more conjugated capsular polysaccharide(s) from *Streptococcus pneumoniae* selected from the group consisting of: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 25 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F.
13. The immunogenic composition of claim 11, wherein the antigen is one or more proteins from *Streptococcus pneumoniae* capable of protecting a host against pneumococcal disease.
- 30 14. The immunogenic composition of claims 9-13, wherein the bleb preparation is derived from a strain which has a detoxified lipid A portion of bacterial LPS, due

to the strain having been engineered to reduce or switch off expression of one or more genes selected from the group consisting of: htrB, msbB and lpxK.

15. The immunogenic composition of claims 9-14, wherein the bleb preparation is  
5 derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to express at a higher level one or more genes selected from the group consisting of: pmrA, pmrB, pmrE and pmrF.
16. The immunogenic composition of claim 3, wherein the antigen is a conjugated  
10 capsular polysaccharide from *H. influenzae* b, and the bleb preparation is from non-typeable *H. influenzae*.
17. The immunogenic composition of claim 3, wherein the antigen is from  
15 *Streptococcus pneumoniae*, and the bleb preparation is from non-typeable *H. influenzae*.
18. The immunogenic composition of claim 17, wherein the antigen is one or more conjugated capsular polysaccharide(s) from *Streptococcus pneumoniae* selected from the group consisting of: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14,  
20 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F.
19. The immunogenic composition of claim 17, wherein the antigen is one or more proteins from *Streptococcus pneumoniae* capable of protecting a host against pneumococcal disease.  
25
20. The immunogenic composition of claim 3, wherein the antigen is from *Moraxella catarrhalis*, and the bleb preparation is from non-typeable *H. influenzae*.
21. The immunogenic composition of claim 20, wherein the antigen is one or more  
30 proteins from *Moraxella catarrhalis* capable of protecting a host against disease caused by *Moraxella catarrhalis*.

22. The immunogenic composition of claims 13-21, wherein the bleb preparation is derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to reduce or switch off expression of one or more genes selected from the group consisting of: htrB, msbB and lpxK.
- 5
23. The immunogenic composition of claims 13-22, wherein the bleb preparation is derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to express at a higher level one or more genes selected from the group consisting of: pmrA, pmrB, pmrE and pmrF.
- 10
24. A vaccine comprising the immunogenic composition of claims 1-23, and a pharmaceutically acceptable excipient or carrier.
25. A method of inducing a faster protective immune response against the antigen contained in the immunogenic composition of claims 1-23, comprising the step of administering to a host an effective amount of the immunogenic composition of claims 1-23.
- 15
26. A method of inducing an enhanced immune response against the antigen contained in the immunogenic composition of claims 1-23, comprising the step of administering to a host an effective amount of the immunogenic composition of claims 1-23.
- 20
27. A method of protecting an elderly patient against a pathogen by administering to said patient an effective amount of the immunogenic composition of claims 1-23 in which the antigen is derived from said pathogen.
- 25
28. Use of the immunogenic preparation of claims 1-23 in the manufacture of a medicament for the treatment of a disease caused by the pathogen from which the antigen is derived.
- 30

29. Use of bleb derived from *Moraxella catarrhalis* as an adjuvant in an immunogenic composition comprising one or more pneumococcal capsular polysaccharides.
30. Use of bleb derived from *Moraxella catarrhalis* as an adjuvant in an immunogenic composition comprising one or more pneumococcal protein antigens.
31. Use of bleb derived from non-typeable *H. influenzae* as an adjuvant in an immunogenic composition comprising one or more pneumococcal capsular polysaccharides.
32. Use of bleb derived from non-typeable *H. influenzae* as an adjuvant in an immunogenic composition comprising one or more pneumococcal protein antigens.
33. A process for making an immunogenic composition comprising the step of mixing an antigen derived from a pathogen which is capable of protecting a host against said pathogen, with an adjuvant comprising a bleb preparation derived from a Gram-negative bacterial strain.



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Figure 1

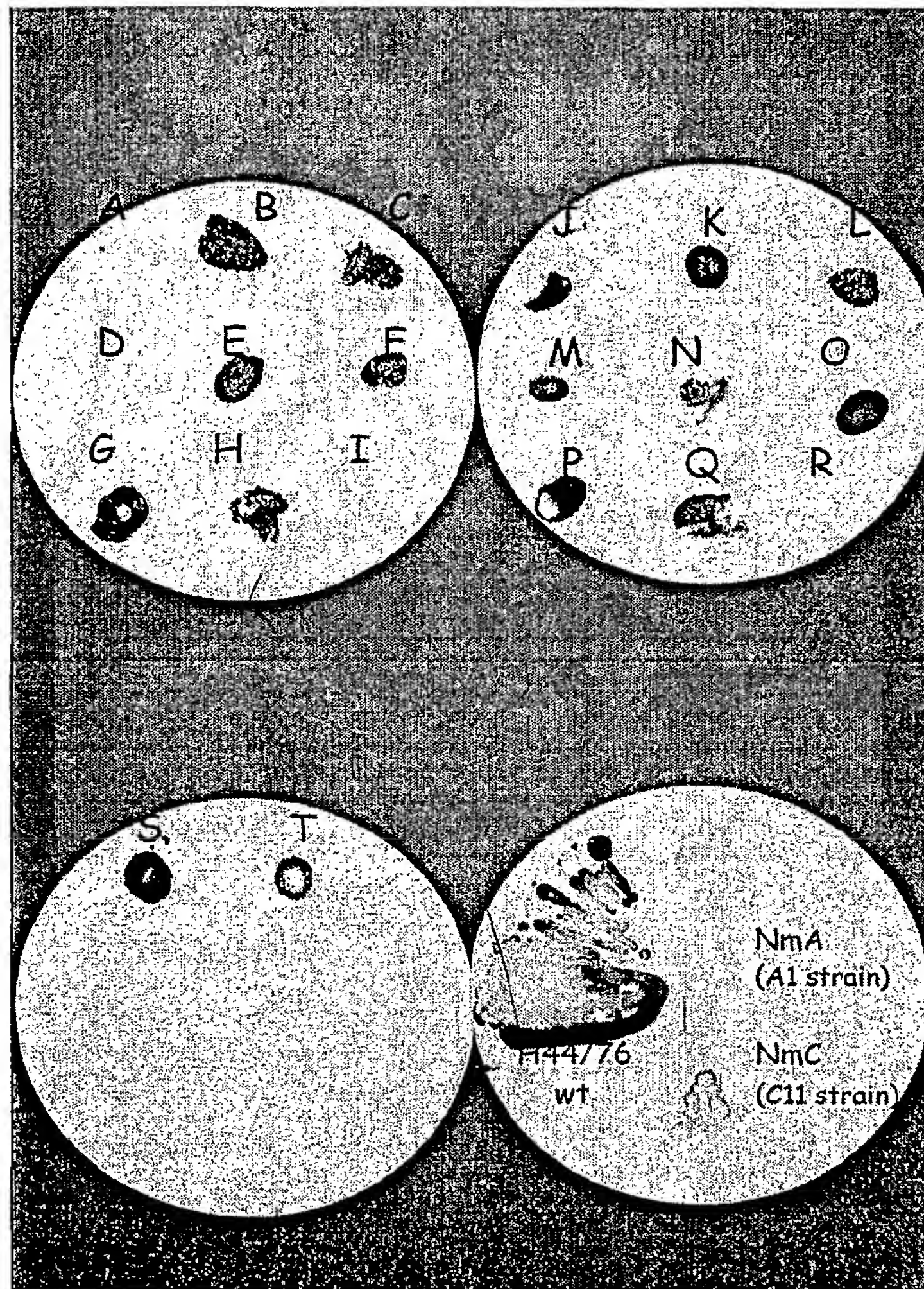


Figure 2

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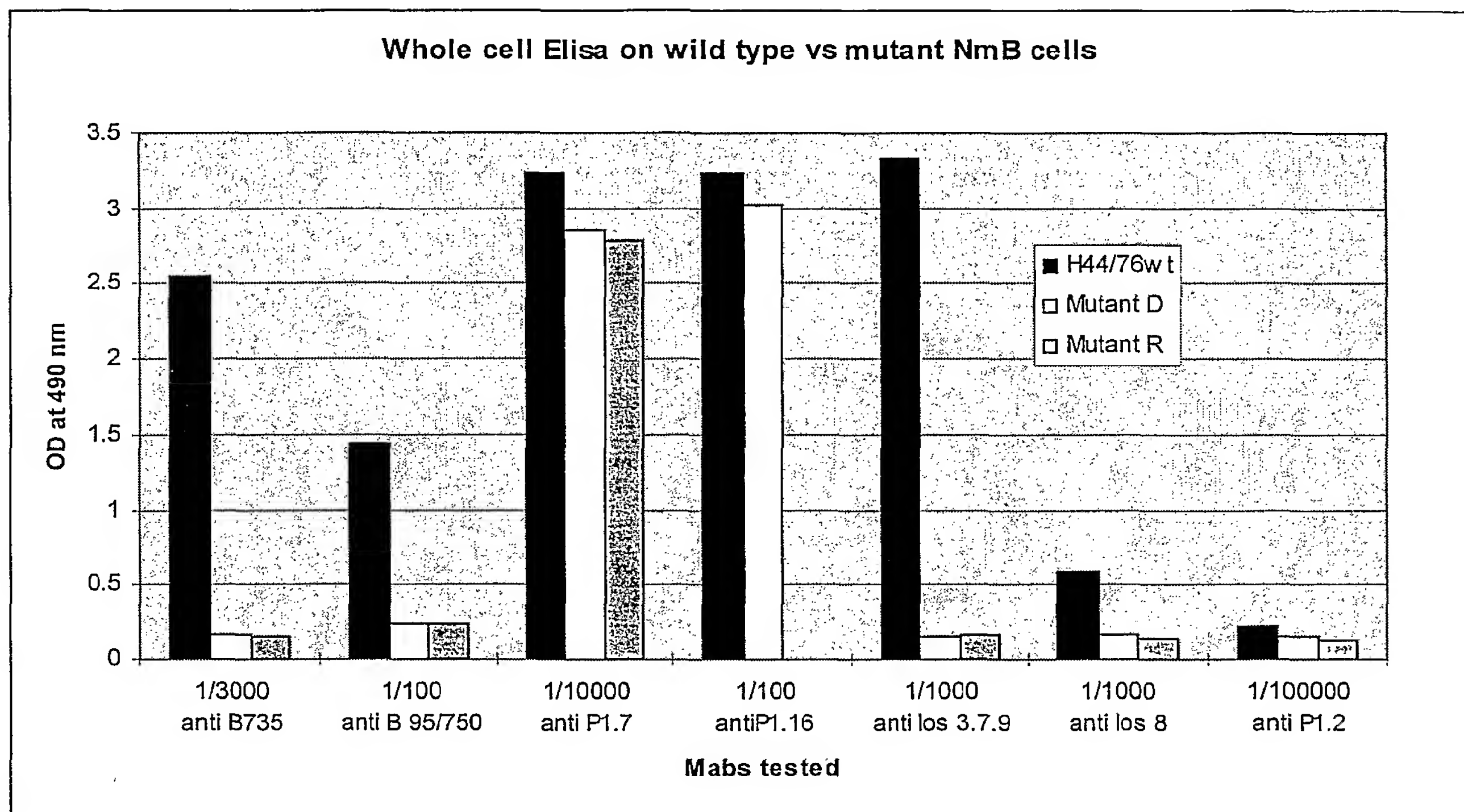


Figure 3

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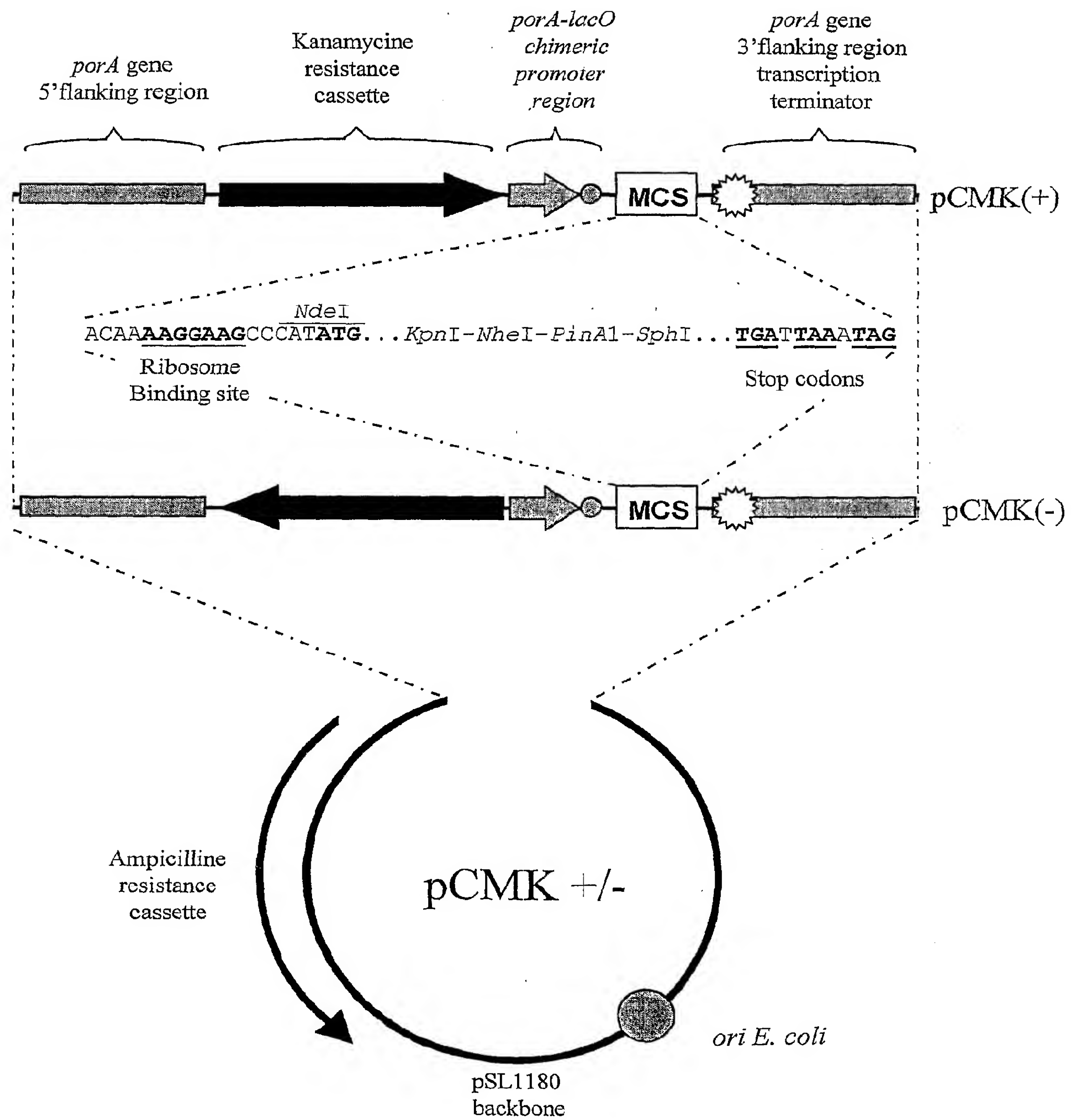


Figure 4

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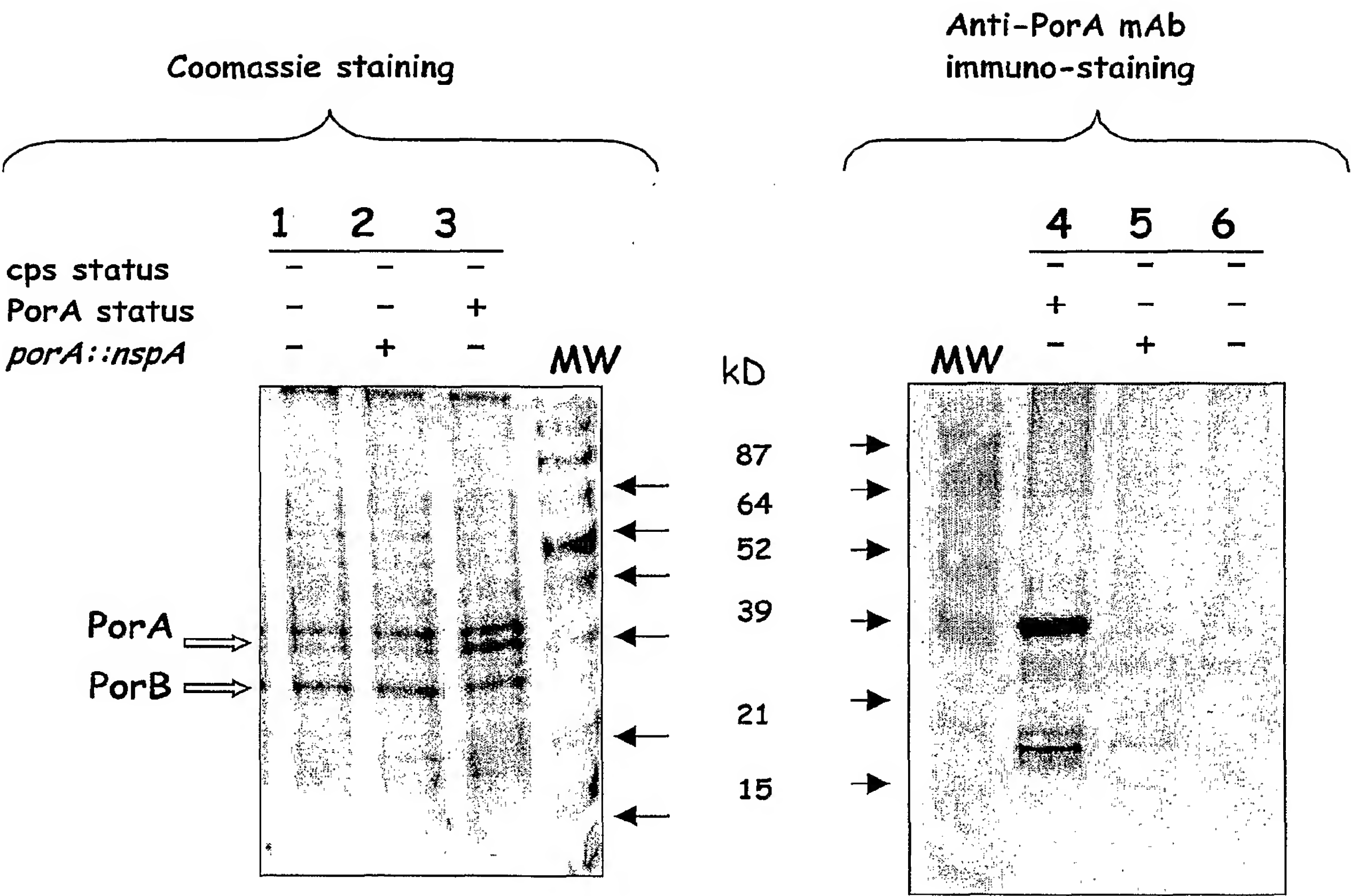




Figure 5

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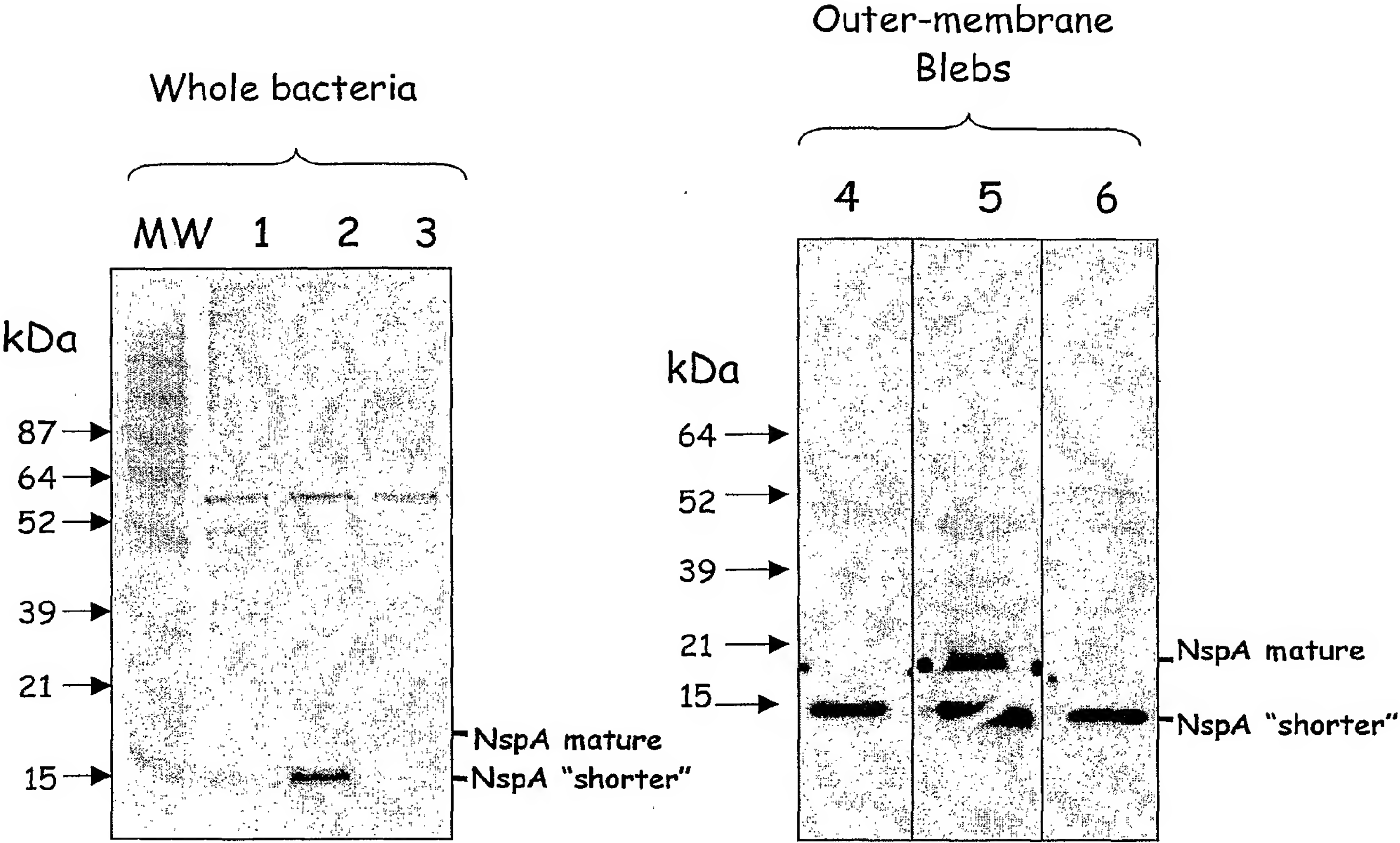
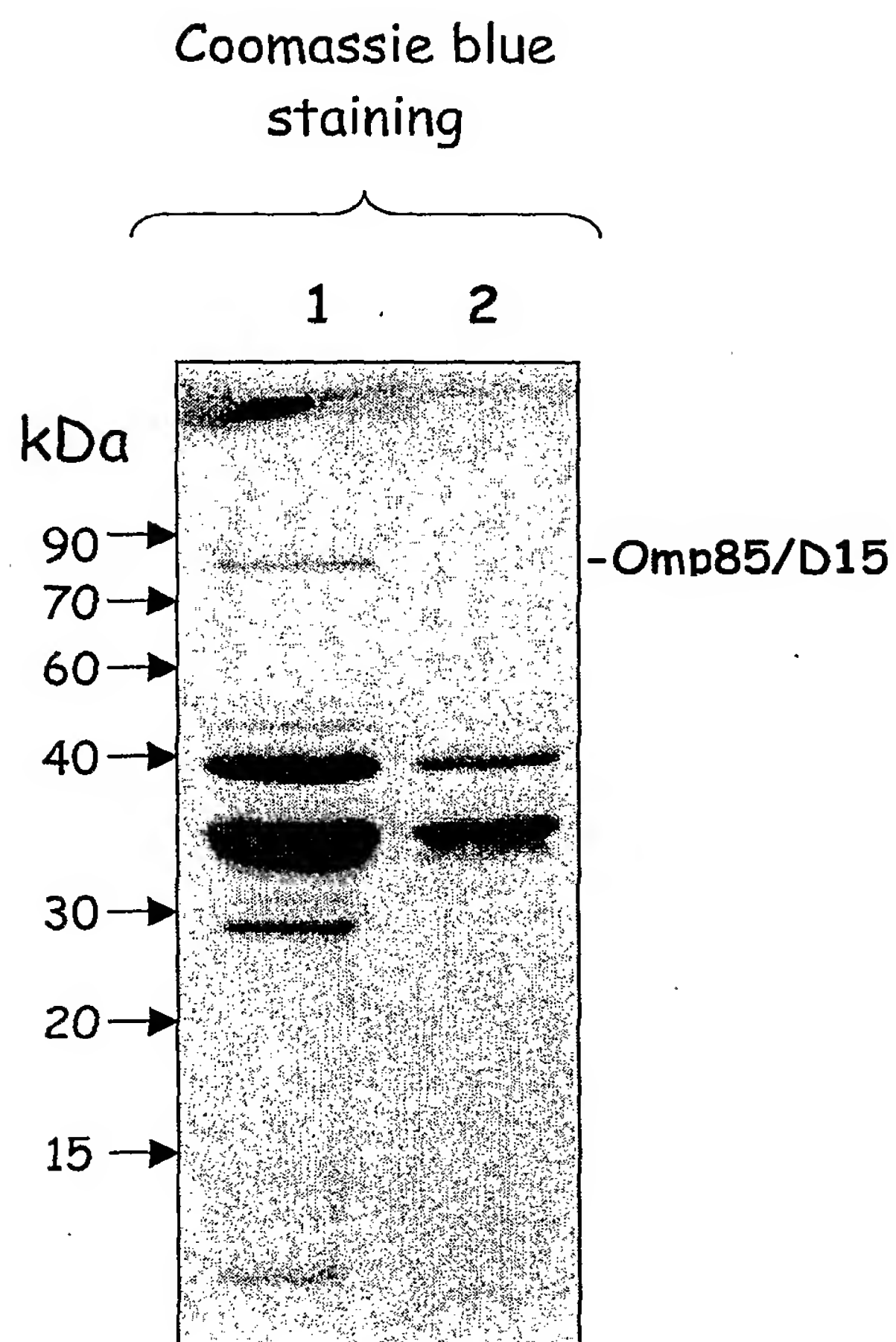


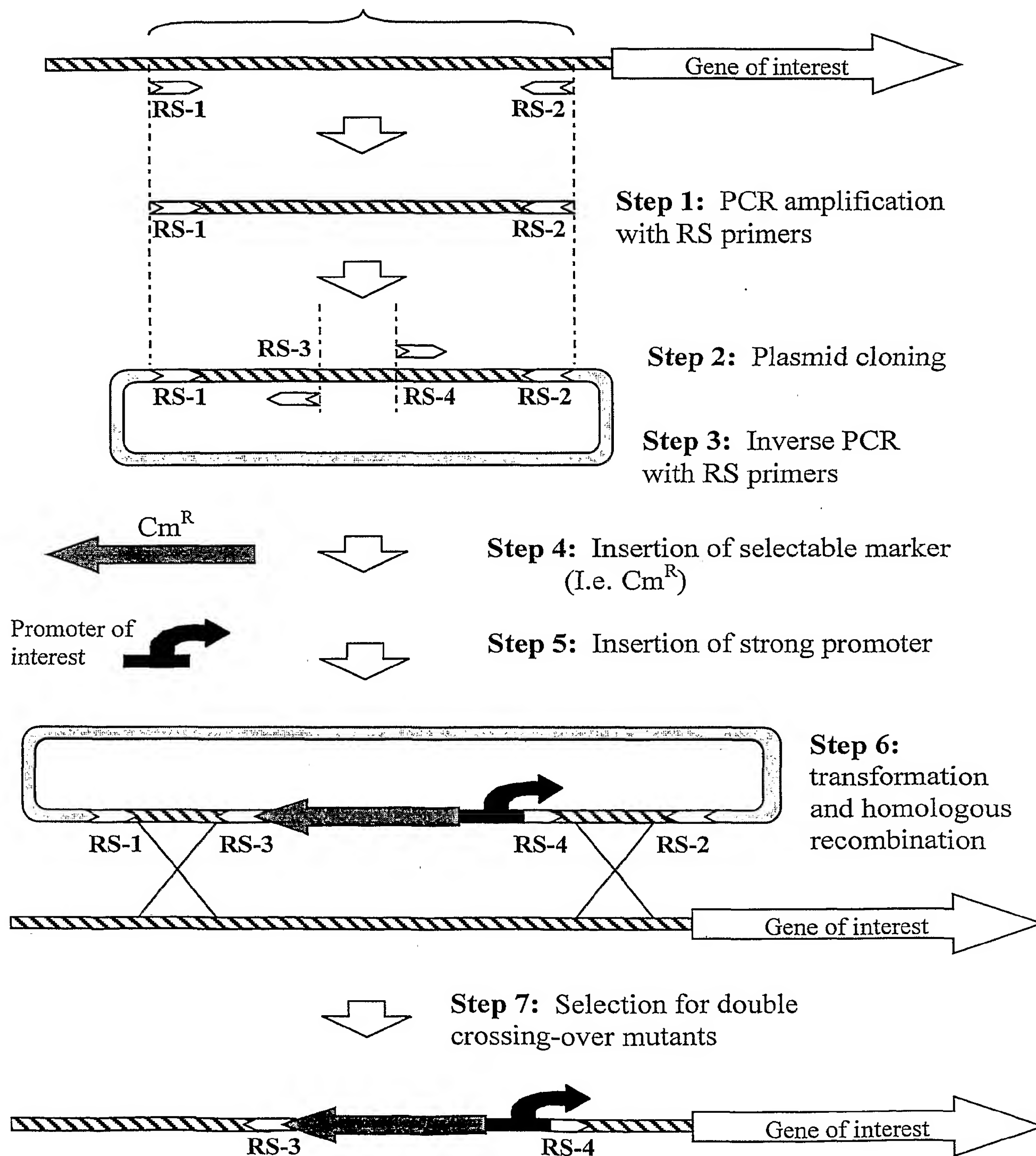
Figure 6

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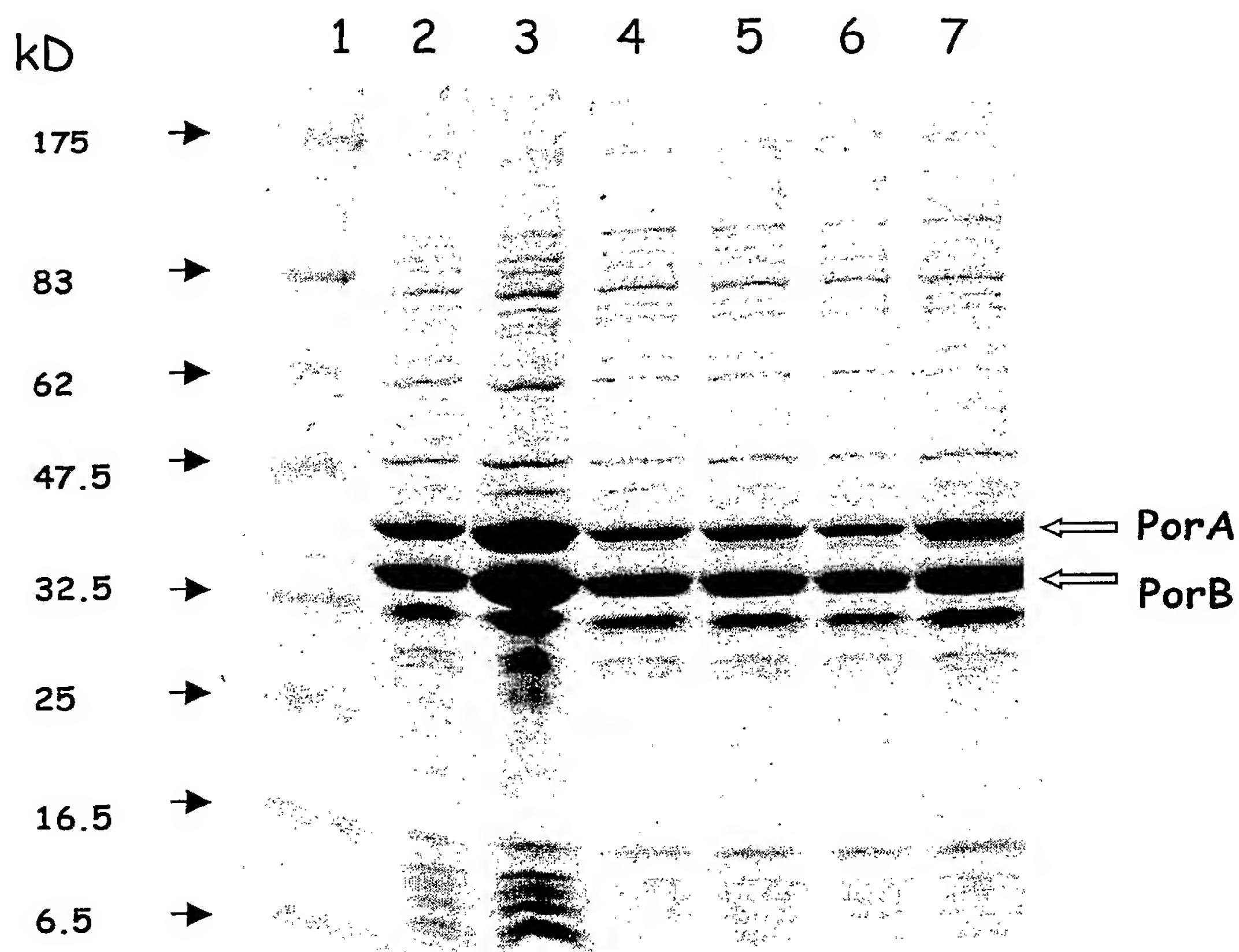
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Figure 7



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Figure 8





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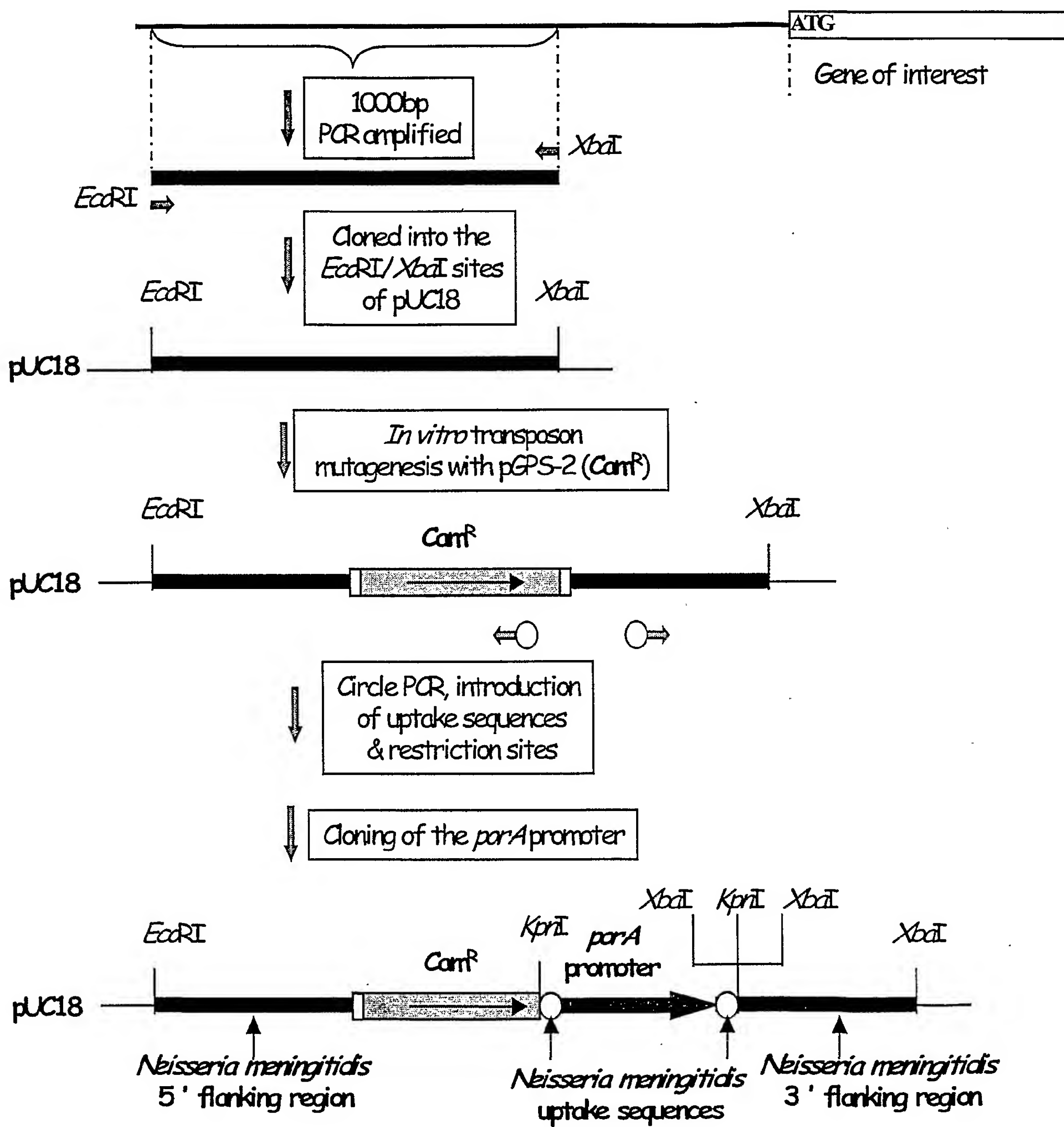
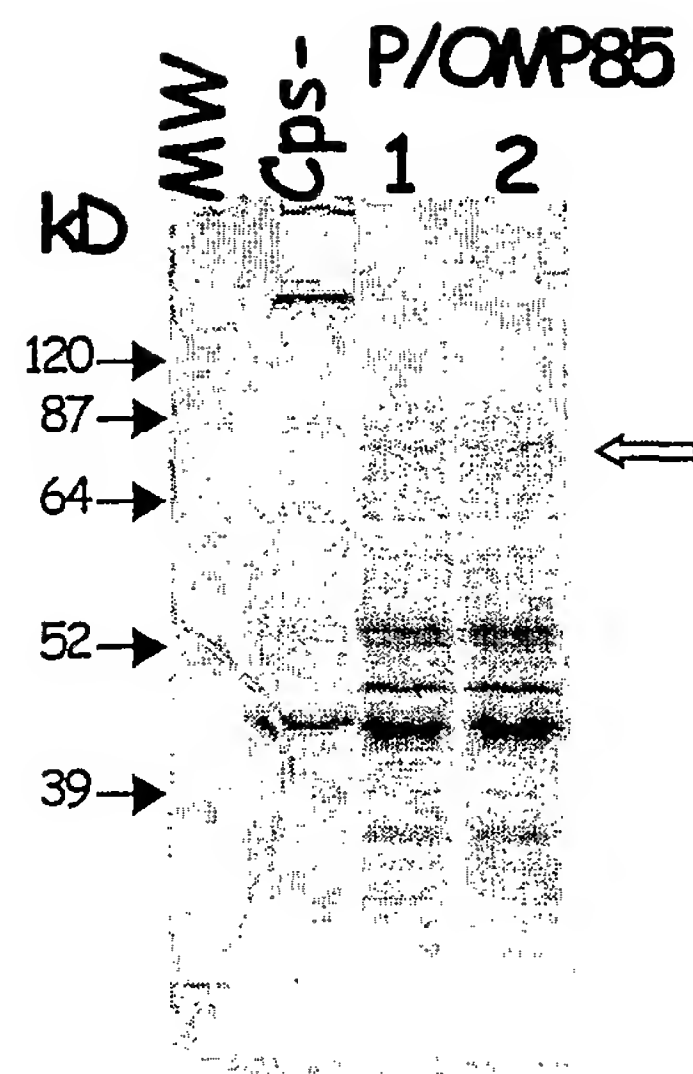
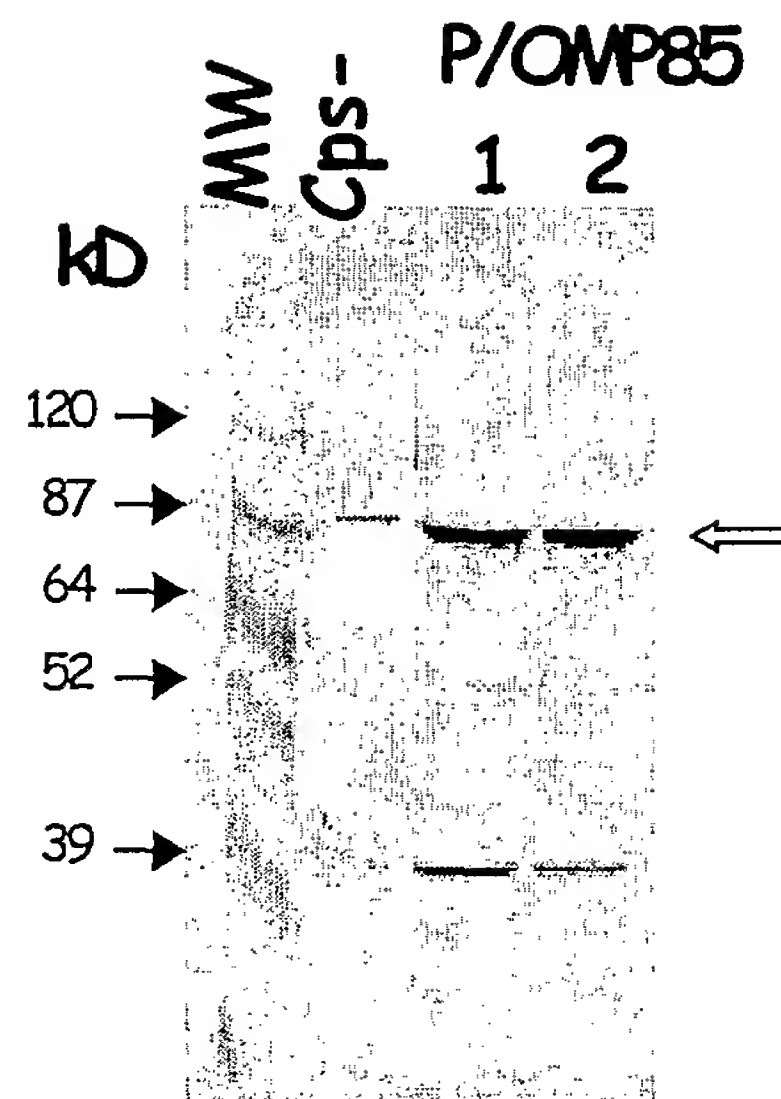


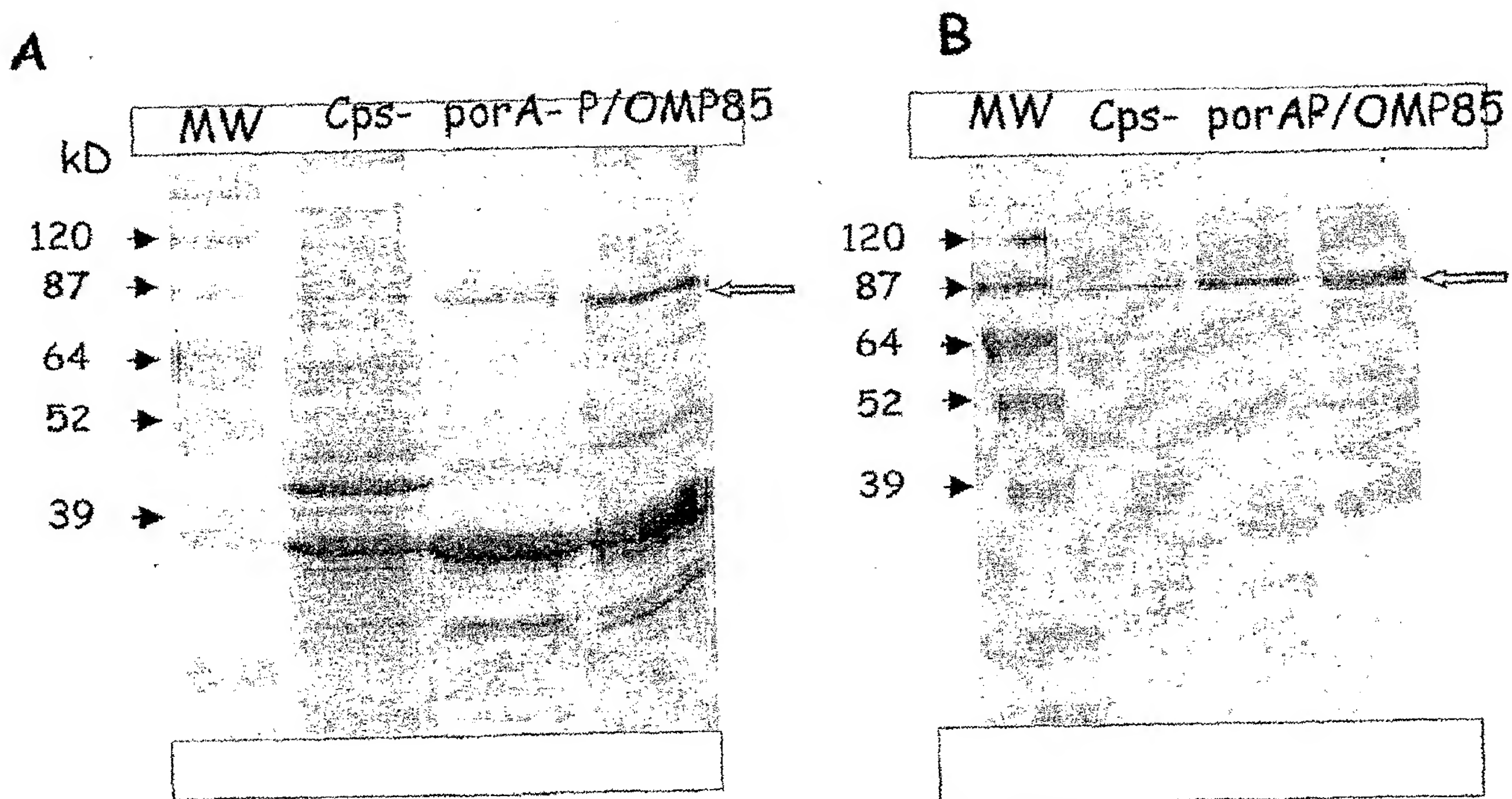
Figure 9

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**A****B****Figure 10**

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Figure 11



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Schematic representation of the recombinant PCR strategy used to delete the *lacO* in the chimeric *porA/lacO* promoter.

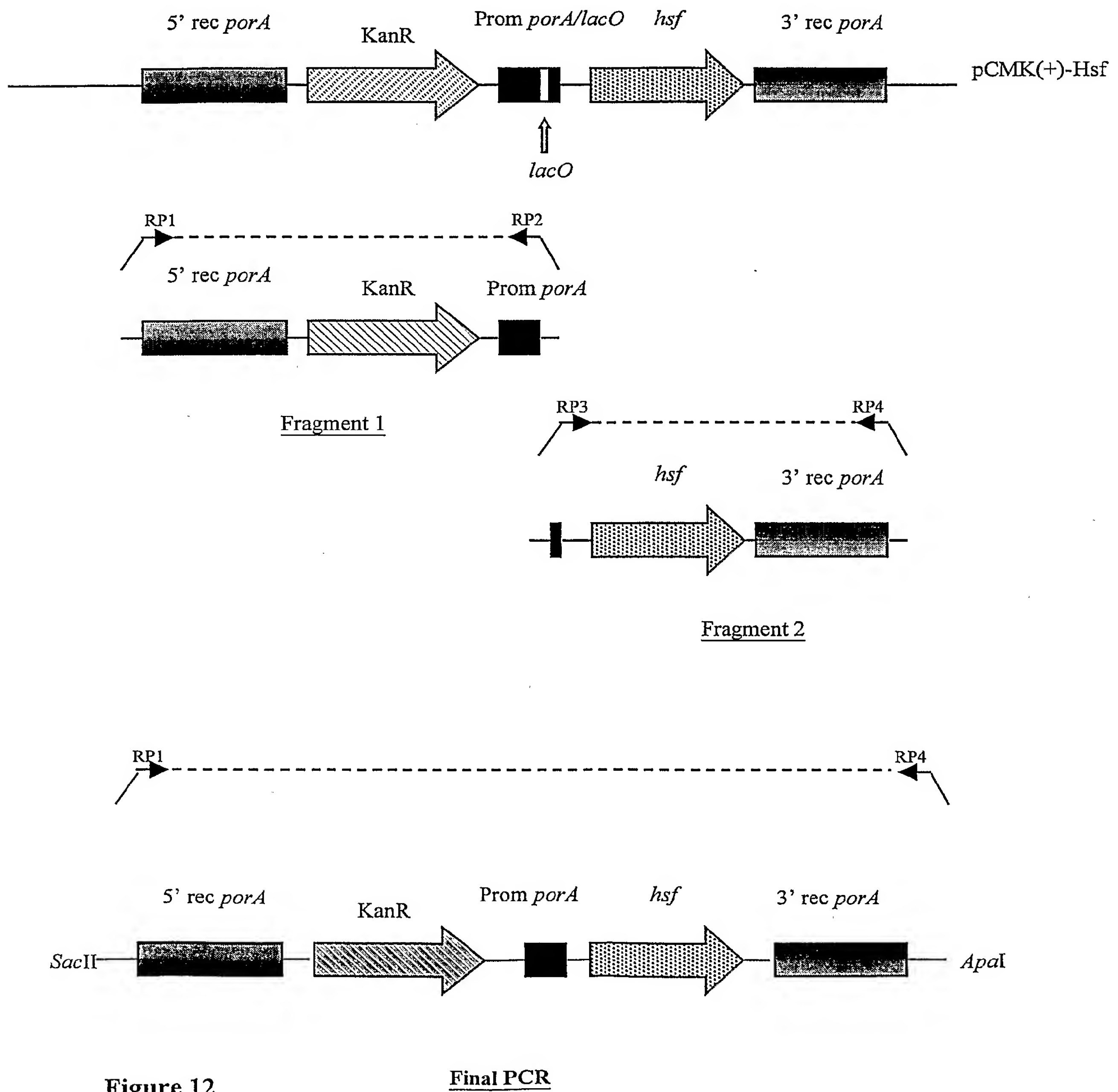


Figure 12

Final PCR



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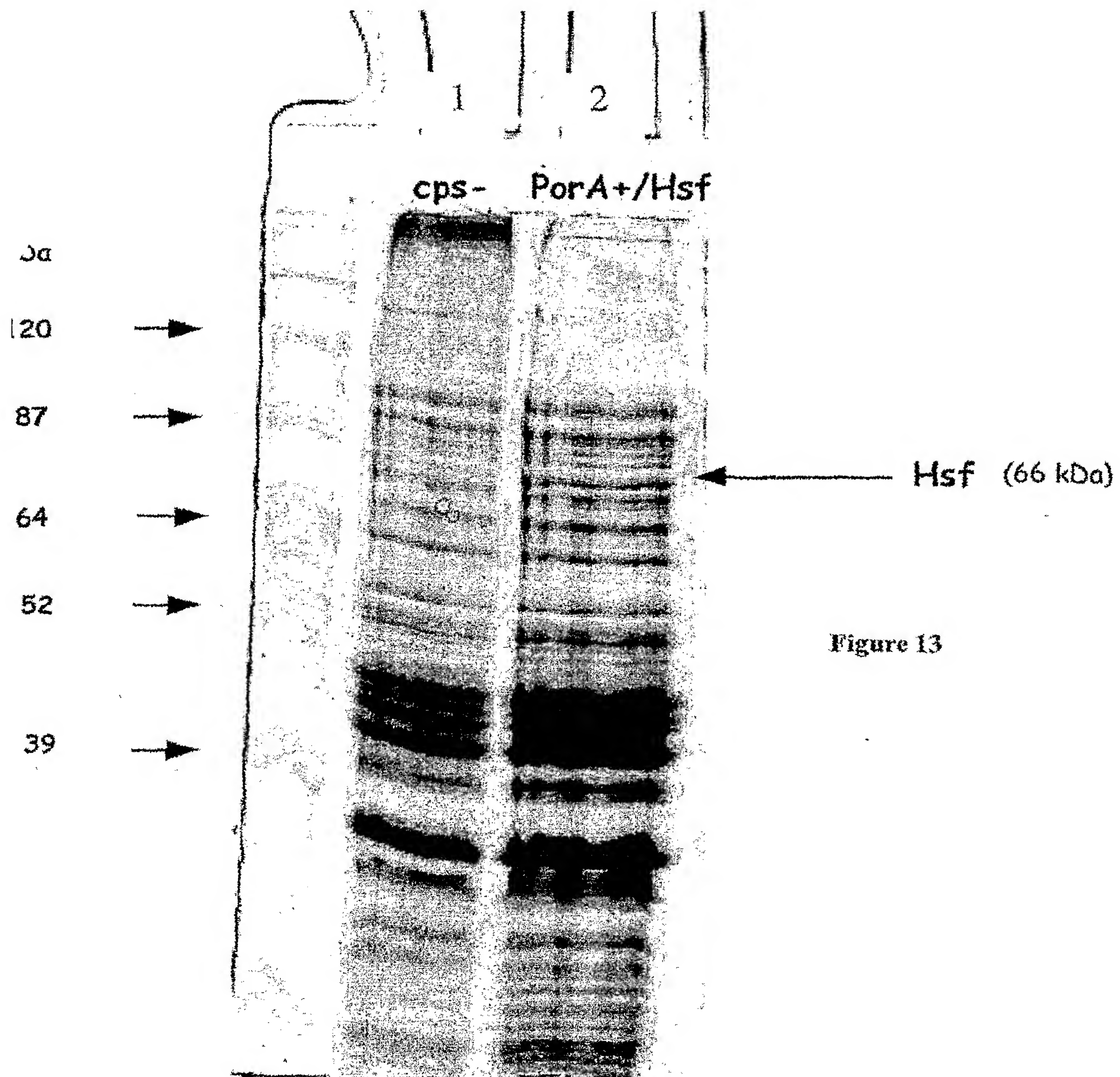
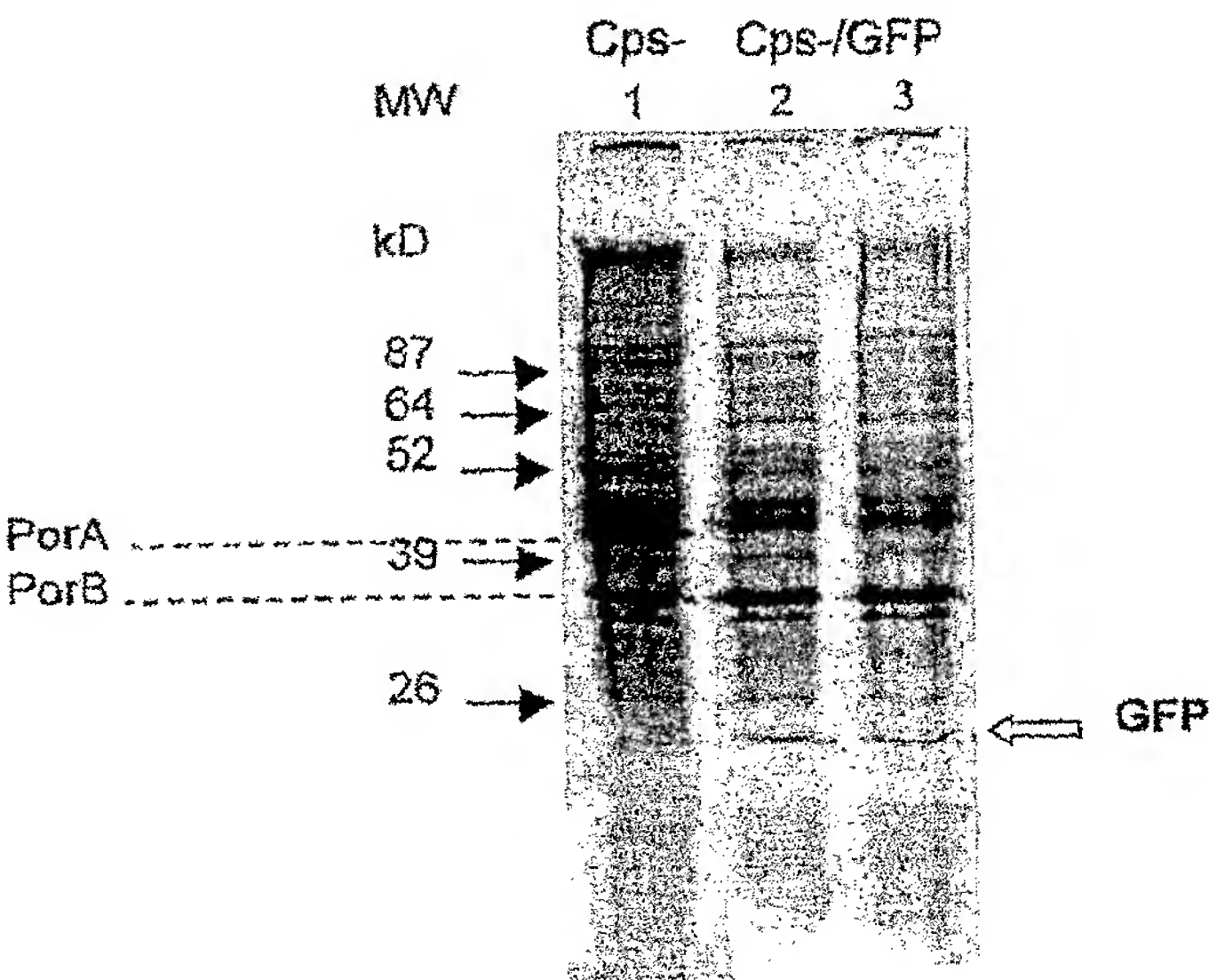


Figure 13

Figure 14



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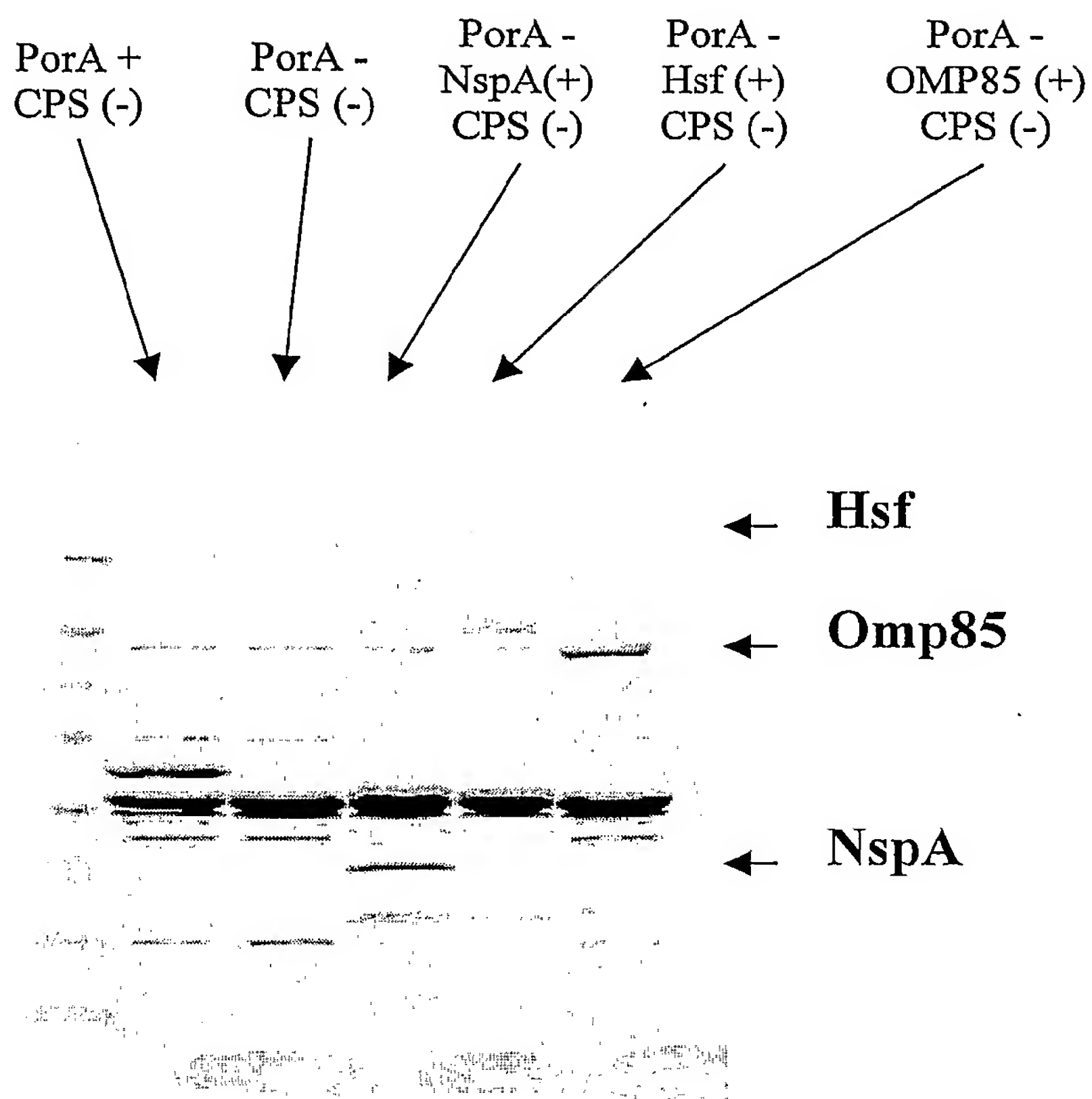


Figure 15

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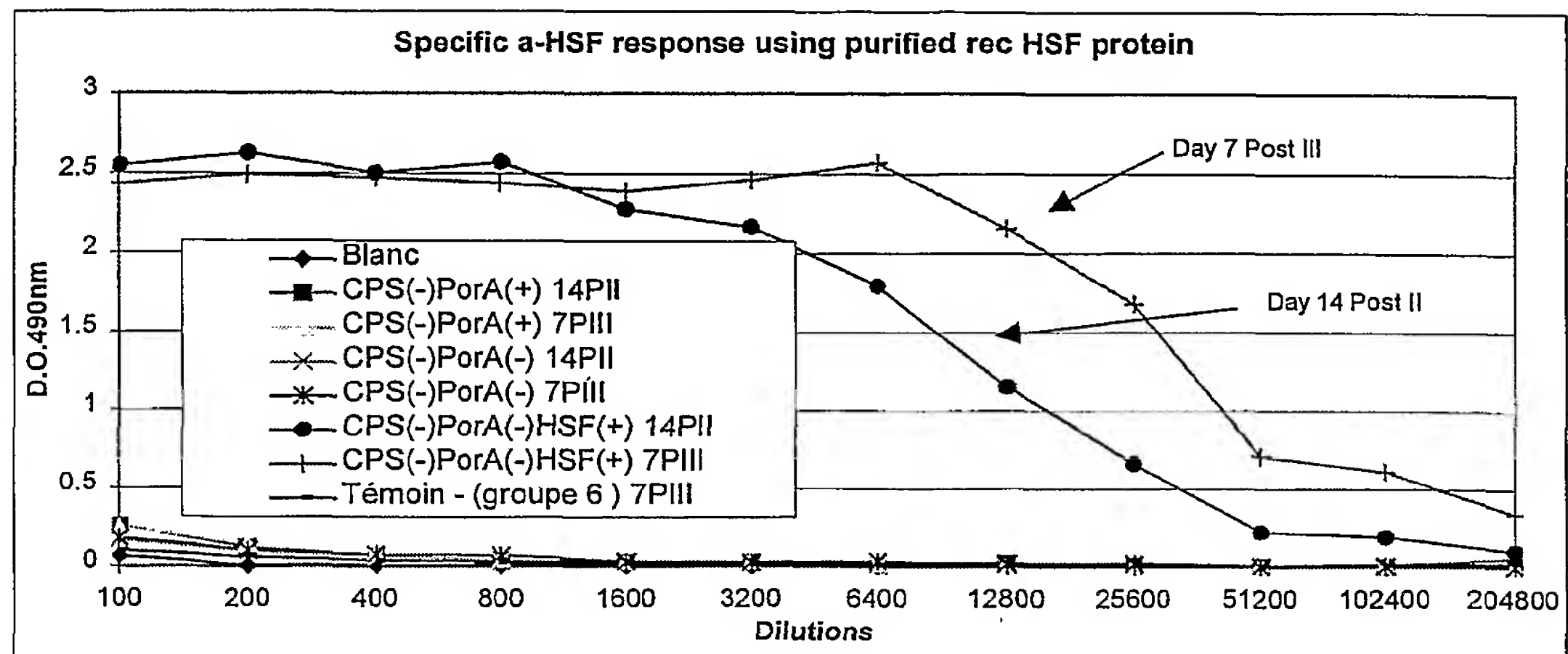


Figure 16



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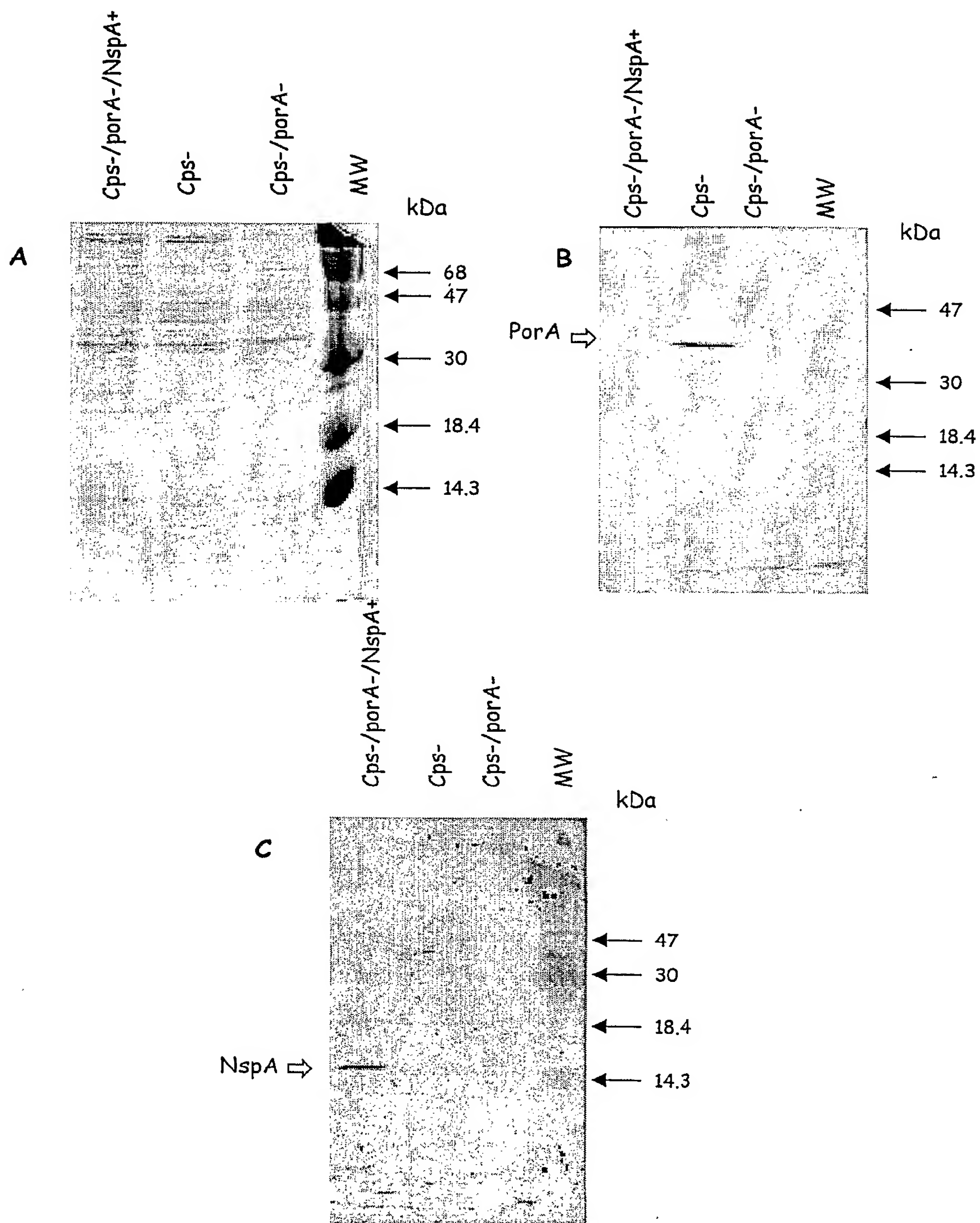


Figure 17